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RETICULOENDOTHELIAL FUNCTION

Methods to evaluate function and correlation
between function and tumor growth -
experimental studies in the rat



Stefan Rydén



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Stefan Rydén

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Title and subtitle

RETICULOENDOTHELIAL FUNCTION. Methods to evaluate function and correlation between function and tumor growth - experimental studies in the rat.

Abstract

A method for evaluation of reticuloendothelial function by the use of a scintillation camera technic was described. Applying this technic, the function of the reticuloendothelial system (RES) was evaluated in rats by measuring the uptake rate into the liver and into the extrahepatic RES of a highly standardized preparation of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid. Estimation of the uptake rates was performed using a two-compartment model. Reticuloendothelial function was determined after the administration of gelatine and methyl palmitate which depressed hepatic colloid uptake rate. Histological and ultrastructural studies of the liver after administration of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and ^{198}Au colloid suggested phagocytosis in Kupffer cells. Compared with $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid, the ^{198}Au colloid accumulated to a greater extent in the bone marrow and less in the spleen and lung. This difference was attributed to a difference in colloidal particle diameter.

Morphological studies indicated an activation of the RES after zymosan administration.

To evaluate if a lysosomal enzyme, beta-hexosaminidase (EC 3.2.1.30), could serve as a marker for reticuloendothelial function, plasma levels of this enzyme were investigated after the intravenous administration of $^{99}\text{Tc}^{\text{m}}$ -sulphur and ^{198}Au colloid, gelatine, methyl palmitate, alcohol and zymosan. An increase of plasma enzyme activity was only observed after zymosan injection. The increase in enzyme levels after zymosan injection was more pronounced in animals with an activated RES than in animals subjected to RES depression by methyl palmitate. Reticuloendothelial function was determined in rats with experimental tumors of different size and site. Hepatic RE function was increased in rats with small tumors and decreased in rats with large tumors. Experimental liver tumors were found to grow faster in animals subjected to a depression of the RES by methyl palmitate at the time of tumor inoculation.

Key words

Reticuloendothelial function; colloids; scintillation camera; lysosomal enzyme; methyl palmitate; zymosan; liver tumor; macrophages; Kupffer cells.

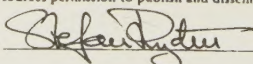
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S-221 85 LUND, Sweden

RETICULOENDOTHELIAL FUNCTION

Methods to evaluate function and correlation

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by

Stefan Rydén

Lund 1983

To Cecilia, Nils and Johan



The thesis is based on the following papers:

- I A SCINTILLATION CAMERA TECHNIQUE FOR MEASUREMENTS OF THE RETICULOENDOTHELIAL FUNCTION. Rydén S, Strand S-E, Palmer J, Stenram U, Hafström Lo, Persson B.
Eur J Nucl Med 7:16-21, 1982.
- II RETICULOENDOTHELIAL FUNCTION IN NORMAL AND TUMOR-BEARING RATS. MEASUREMENTS WITH A SCINTILLATION CAMERA TECHNIQUE. Rydén S, Bergqvist L, Hafström Lo, Strand S-E.
Eur J Ca Clin Oncol 19:965-970, 1983.
- III RELEASE OF β -HEXOSAMINIDASE AFTER ADMINISTRATION OF DIFFERENT AGENTS AFFECTING THE RETICULOENDOTHELIAL SYSTEM. AN EXPERIMENTAL STUDY IN RATS. Rydén S, Bergqvist L, Hafström Lo, Hultberg B.
Submitted for publication.
- IV INFLUENCE ON PLASMA β -HEXOSAMINIDASE OF RETICULOENDOTHELIAL STIMULATION AND DEPRESSION. AN EXPERIMENTAL STUDY IN RATS. Rydén S, Hultberg B, Hafström Lo, Isaksson A.
Submitted for publication.
- V INFLUENCE OF A RETICULOENDOTHELIAL SUPPRESSING AGENT ON LIVER TUMOR GROWTH IN THE RAT. Rydén S, Bergqvist L, Hafström Lo, Hultberg B, Stenram U, Strand S-E.
Submitted for publication.

In the text these papers are referred to by their Roman numerals.

CONTENTS

1.	INTRODUCTION	5
1.1.	Historical background	5
1.2.	Definitions	6
1.3.	Origin of Kupffer cells	7
1.4.	Morphology of Kupffer cells	7
1.5.	Function of Kupffer cells	8
1.6.	Opsonins	8
1.7.	Physiological and pathophysiological involvement of the reticuloendothelial system	9
1.8.	Measurements of reticuloendothelial function	11
1.9.	Depression of reticuloendothelial function	15
1.10.	Stimulation of reticuloendothelial function	16
1.11.	Lysosomal enzymes and reticuloendothelial function	16
1.12.	Reticuloendothelial function and tumor growth	17
2.	PURPOSE AND AIM OF THE STUDY	19
3.	MATERIALS AND METHODS	20
3.1.	Animals	20
3.2.	Test substance	20
3.3.	Measurements of reticuloendothelial function	22
3.3.1.	Scintillation camera technique	22
3.3.2.	Computer analyses	23
3.4.	Tumors	25
3.5.	Morphological studies	26
3.6.	Preparation of other agents	26
3.7.	Enzyme analyses	28
3.8.	Statistical analyses	28
4.	RESULTS	29
5.	DISCUSSION	46
5.1.	Methodological considerations	46
5.2.	Reticuloendothelial function and tumor growth	53
5.3.	Clinical considerations and future aspects	55
6.	SUMMARY AND CONCLUSIONS	57
7.	ACKNOWLEDGEMENTS	58
8.	REFERENCES	59

"... in the reticuloendothelial system a guiding thought is offered, the critical application of which will bring fruitful results in the various fields of medicine."

L Aschoff 1924

1. INTRODUCTION

1.1. Historical background

The reticuloendothelial system (RES) is a system of cells, scattered in the body, in close contact with the blood which has the ability of phagocytosis as a common denominator (1). When Aschoff in 1924 gave a name to this system, his work was preceded by a number of important discoveries. Documented attempts at vital staining were performed as early as 1567 when Missaud fed madder to animals in order to study their bones (2). Deliberate vital staining procedures were performed by von Gleichen-Russworm in 1778 (3) and more systematically by Ehrenberg in 1838 (4), who first described intracellular acid vacuoles, later described as lysosomes.

The early use of "Chinese (India) Ink" could be exemplified by the studies of uptake in frog white blood cells by von Recklinghausen in 1863 (5).

Ponfick (1869) demonstrated the important role of the liver and spleen in the uptake of the extraneous particles of dye, the first experimental study to give an indication of a "system" of phagocytic cells (5). Although Wedl (1854) had described pigment carrying cells of the liver (6), Karl von Kupffer (1876) is credited with the discovery of these cells (7). Kupffer observed that certain cells in the liver stained black with gold chloride. He described them as "dark black stars ... always in touch with a capillary ... (but) also closely associated with liver cells" (7).

Further confirmation of the phagocytic nature of these cells was made

by Asch in 1884 (8) who studied the liver uptake of dye and fat from the blood stream.

It was Ilja Metchnikoff's discovery in the 1880s that lay the groundwork for the theory of phagocytosis (9). Metchnikoff showed that, as a feature of host defense, in addition to the free wandering macrophages in the blood and lymph, fixed cells were present in many organs. All of these could engulf small particles by extending pseudopods. It was already obvious to von Kupffer that these cells were true intravascular components. Aschoff (1) regarded these cells as specialized endothelial cells and named these scattered fixed macrophages: "reticuloendothelial cells".

1.2. Definitions

Although Aschoff's terminology has been challenged, it has gained general acceptance. The term "macrophage system", which may be more appropriate, has often been used synonymously. This system includes sessile and wandering macrophages and excludes polymorphonuclear leukocytes or microphages. A schematic presentation is given in Figure 1 according to Saba (10).

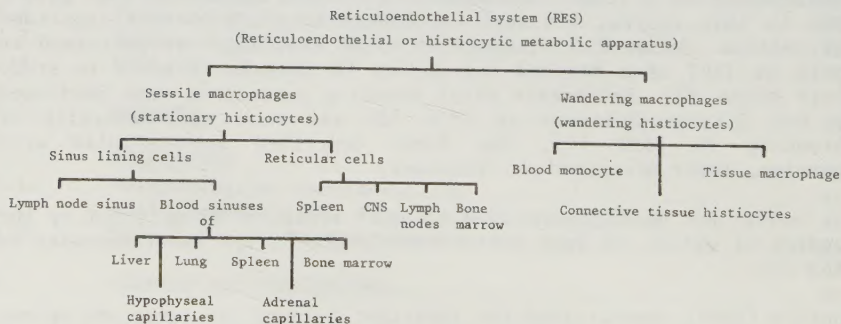


Fig 1. Slightly modified version of Aschoff's concept of RES (10).

Of primary interest in this study are the fixed macrophages of the liver, spleen, lung and bone marrow, which have been found to be primarily responsible for the clearance of particulate matter from the blood. The macrophages of the liver and the spleen manifested approximately 85-90 % of the total intravascular phagocytic activity (10). Kupffer cells constituted about 80-90 % of the fixed macrophages of the RES (11).

1.3. Origin of Kupffer cells

Kupffer cells, as well as macrophages in the lung, the spleen and lymph nodes, derive from a common precursor in the bone marrow (12). The stem cell may be capable of becoming either a granulocyte or a macrophage (13). From the blood, monocytes enter the tissue and further differentiate into macrophages (14). Experiments with radiolabeled bone marrow cells have shown that the major increase in the number of liver macrophages was due to recruitment of cells from the bone marrow (14). However, recent evidence suggested that Kupffer cell proliferation may occur in the liver (15).

1.4. Morphology of Kupffer cells

Ultrastructural studies of the liver have contributed much information about the morphology and function of liver sinusoidal cells. Four cell types line hepatic sinusoids: Kupffer cells, endothelial cells, fat storing (Ito) cells and pit cells (16). The Kupffer cells were found lining the lumen of hepatic sinusoids, particularly around the portal tract often protruding into the sinusoids (17). They had the morphologic features typical of macrophages with bulky cytoplasm containing many well-developed lysosomes and pinocytotic vesicles (16). Under normal physiologic conditions, digested material was rarely seen in lysosomal vacuoles, suggesting that the cells were in a resting state (18). The cell coat of Kupffer cells consisted of two layers: a) a thin layer close to the plasma membrane, and b) a 50-70 nm thick filamentous or fuzzy outer layer (19). It has been suggested that a receptor for the Fc-region of IgG was located in or below the thin coat and that a receptor for the conversion product of the third component of complement, C3b, was located in the outer filamentous layer (20).

Kupffer cells possessed three structures that were related to pinocytosis: a) bristle-coated micropinocytotic vesicles, with a diameter of about 100 nm, b) the thick fuzzy coat of the cell surface, and c) worm-like structures protruding from the cell surface (16).

The opsonic protein, fibronectin (21), may interact with the fuzzy coat, a finding which was consistent with the observation that this structure stains for glycoprotein (19).

1.5. Function of Kupffer cells

Macrophages have been shown to possess the ability to endocytose large quantities of materials in the circulation, such as bacteria, tissue debris and foreign particles (10), much of which is continuously produced in small quantities under normal conditions (22). Endocytosis was achieved by pinocytosis and phagocytosis.

The abundance of the above mentioned structures associated with pinocytosis suggested that Kupffer cells continuously pinocytose plasma under normal conditions (18). Lysosomal enzymes (23) and gut derived endotoxin (24) probably were cleared by the liver by receptor-mediated pinocytosis. Phagocytosis in vivo was initiated by the recognition and attachment of particulate material to the cell surface. Foreign colloidal particles have been found attached to the Kupffer cell membrane soon after intravenous injection (16, 25, 26). Depending upon the composition of particulate material, it may be degraded by lysosomal enzymes or stored in lysosomes (15). Thus protein aggregates may have been digested, in contrast to inert particles which were stored in the phagocytes. Hepatic blood flow was of fundamental importance for Kupffer cell phagocytosis. Adequate perfusion of the sinusoids with portal blood seemed to be a prerequisite for efficient phagocytic function (27). The recognition of appropriate material for phagocytosis was mediated in the circulation by circulating recognition factors (opsonins) and at the cellular level by cell surface receptors (15).

1.6. Opsonins

The importance of serum factors was already acknowledged by Metchnikoff who suggested the name "stimulins" (28). Wright and Douglas (1903) demonstrated that there existed in normal human serum a substance which stimulated phagocytosis by leukocytes (29). They termed this substance "opsonin" (greek: "opsono" = I cater for). Hektoen demonstrated in 1908 that specific human recognition factors existed for leucocyte phagocytosis of various bacteria (30).

Depression of reticuloendothelial (RE) function has been associated with a decrease in serum opsonins (31, 32). A technique of evaluating opsonin activity was described by Saba and DiLuzio (33) studying the in vitro phagocytosis of gelatinized RE-test lipid emulsion by rat liver slices. Kupffer cells of normal rat liver in an incubation medium consisting of normal rat serum and heparin manifested a 17-fold enhancement in colloid uptake as compared to the degree of phagocytosis manifested in buffer medium (34). Pisano demonstrated that human serum was as effective as rat serum in promoting rat liver slice phagocytosis (35).

A large molecular weight (MW 800 000 at 4°C, 400 000 at 37°C) α -2-globulin was isolated and purified from human serum (36). This protein (α -2-macro globulin, α -2-opsonic protein) that coated foreign

particles and augmented Kupffer cell phagocytosis has been shown to be identical to cold insoluble globulin or fibronectin (37, 38, 39). The α -2-opsonic protein has been determined by electroimmunoassay. Depletion of the protein has been correlated with decreased liver RES phagocytic activity (21).

This opsonic protein has been considered as non-immunospecific in contrast to the immunospecific IgG and IgM antibodies which recognize specific antigenic material (15).

Foreign or effete endogenous particles, when exposed to blood, were coated with molecules of opsonin, particularly fibronectin (40). The plasma level of fibronectin appeared to correlate directly with Kupffer cell non-immunospecific phagocytic capacity (41). Immunospecific, IgG or IgM, mediated phagocytosis was receptor-specific via Fc-receptors and C3b-receptors, respectively (42).

1.7. Physiological and pathophysiological involvement of the reticuloendothelial system

Numerous studies during the last decades have documented an involvement of the reticuloendothelial system in many physiological and pathophysiological events, the most important of which are listed in Table I.

Table I. Involvement of reticuloendothelial function

Metabolism

Carbohydrate metabolism	Filkins 1975 (43), 1980 (44), 1981 (45)
Protein metabolism	Hyman & Paldino 1960 (46)
Lipid metabolism	DiLuzio 1960 (47) Stein et al 1969 (48) Blackburn 1977 (49)
Iron metabolism	Vannotti 1957 (50) Fillet et al 1974 (51)

Infection

Biozzi et al 1957 (52)
Rogers 1960 (53)
Pardy et al 1977 (54)

Immunological processes

Triger 1976 (55)
Diamond 1982 (12)

Shock

Fine 1958 (56)
Zweifach 1960 (57)
Saba 1975 (58)
Wolter et al 1978 (59)

Hemostasis

Kaplan & Saba 1978 (60)
Kaplan 1981 (61)

Tumor growth

Old et al 1960 (62)
Levi & Wheelock 1974 (63)
DiLuzio 1975 (64)
Roos & Dingemans 1977 (65)
DiLuzio 1981 (66)

Lysosomal enzyme secretion and clearance

Stahl et al 1976 (67)
Schlesinger et al 1978 (68)
Page et al 1978 (69)

1.8. Measurements of reticuloendothelial function

From a physiologic point of view the RES could be characterized in terms of its phagocytic behavior. This has lead to the development of the "colloid clearance technique" for studying RE function. The kinetics associated with the vascular clearance of colloids was first investigated in a quantitative manner by Maxfield and Mortensen (1941) using thorium dioxide (Thorofrast) (70).

Systematic studies of the clearance of colloidal carbon (India Ink) from the blood were performed by Halpern et al (71). However, the results of those early studies were vitiated by the toxic reactions caused by the shellac present in the India Ink preparations.

With a carbon suspension in fish glue free from shellac (particle size about 25 nm), no toxic side effects were noted (72).

Quantitative studies of vascular carbon clearance in the rat allowed for the application of mathematical expressions to phagocytosis (72). These investigators emphasized the exponential nature of the disappearance curve. After the injection of carbon the concentration of carbon in the blood could be expressed by Equations 1a and 1b:

$$C(t) = C(0) \cdot 10^{-kt} \quad (1a)$$

or

$$k = \frac{1}{t} \{ \log C(0) - \log C(t) \} \quad (1b)$$

where $C(t)$ was the concentration of carbon in the blood at the time (t) and $C(0)$ was the blood concentration just after the injection and before the particles were absorbed by the RES. The constant k characterized the rate of clearance and was called the "granuloplectic (or phagocytic) index" (72). It was found that, by increasing the dose of carbon, the granuloplectic index tended to decrease, and above a certain dose ("the critical dose") the granuloplectic index was inversely proportional to the dose of carbon injected, which led to Equation 2:

$$k \cdot D = Q \quad (2)$$

where k was the granuloplectic index and D the dose of carbon injected. Q was a constant and was found to equal 0.208. With small doses of colloid, blood clearance was extremely rapid and the colloid uptake was almost completely confined to the liver (72).

Contemporary studies by Dobson and Jones (1952) on the blood clearance of radioactive chromic phosphate further emphasized the significance of the "critical colloid dose" and the value of the half time ($t_{1/2}$) associated with the removal of tracer doses of colloids (below the

critical dose) in the calculation of the fractional clearance rate (k) for estimation of liver blood flow (73). Provided that, after injection of minute doses of colloid, the particles were taken up almost completely during their first passage through the liver and that particles were not taken up by any other organ, the blood clearance was a measure of liver blood flow. This formed the basis for the "fractional clearance rate K" (10, 73, 74, 75). The "fractional clearance rate" could be calculated according to Equation 3:

$$K = \frac{\ln 2}{t_{1/2}} \quad (3)$$

Above the critical dose, colloid particles were not completely absorbed through their first passage through the liver, and the value of k was more a measure of phagocytic activity provided blood flow was normal (76). It was indeed observed that by increasing the dose, the colloid uptake by the extrahepatic RES, mainly the spleen, increased (72).

The impact of relative liver and spleen size on the rate of colloid clearance was also explored by Biozzi et al (72). A cubic relationship between the relative liver and spleen size and RE activity was demonstrated, which rendered comparisons between different species possible. The term "corrected phagocytic index", alpha (α), as a comparative index of RE functional activity between different species was generated. This term (α) was related to the phagocytic index (k) by the Equation 4:

$$\alpha = \sqrt[3]{k \cdot \frac{W}{WLS}} \quad (4)$$

where W represented body weight and WLS the combined weight of the liver and spleen (72).

The importance of particle size on the clearance rate was recognized early. Large particle colloids disappeared rapidly from the blood and were found primarily in the liver and spleen, whereas smaller particles were cleared from the blood at a lower rate and were found to a greater extent in the bone marrow (77). These findings have been confirmed by Scott et al (78) using radioactive colloidal gold.

Benacerraf et al (79) have described some of the characteristics of a good colloid preparation, used for measuring RE activity:

1. the particles should be phagocytized by the cells of the RES in contact with the blood;
2. they should not normally cross the capillary barrier;
3. they should be homogenous in size;
4. the preparation must be stable in the blood at the concentration used;
5. the substance should be non-toxic for RE cells and for the organism;
6. the substance should be accurately measurable in the blood and in the tissues by chemical or physical methods. It should be able to be traced histologically in the cells of the RES;
7. it should not be taken up by other cells than those investigated.

Numerous particle preparations have since been used for studying RE function, some of which are summarized in Table II.

$^{99}\text{Tc}^{\text{m}}$ -sulphur colloid has been used extensively for estimation of RE function (85, 91, 92) and for routine liver scan (93).

The development of radiolabeled compounds and scintillation camera techniques has permitted dynamic non-invasive studies of the uptake of the radioactivity in different organs (81, 94, 95, 96).

Table II. Colloid particle preparations used for determination of RE function.

Particle preparation	Particle size (nm)	Reference
Colloidal carbon	~ 25	Biozzi et al 1953 (72)
Saccarated iron oxide	~ 2.5	Benacerraf et al 1957 (79)
^{32}P -Chromium phosphate		"
^{131}I -Heat denatured albumin		Benacerraf et al 1957 (74)
^{125}I -Microaggregated albumin	< 220	Briner 1968 (80)
$^{99}\text{Tc}^{\text{m}}$ -Albumin microparticles	500-2 000	Reske et al 1981 (81)
$^{99}\text{Tc}^{\text{m}}$ -Microaggregated albumin	30-100	McAffee et al 1982 (82)
^{198}Au -colloidal gold	~ 35	Scott et al 1967 (78)
"	~ 4	"
^{131}I RE-Test lipid emulsion		DiLuzio & Riggi 1964 (83)
$^{99}\text{Tc}^{\text{m}}$ -sulphur colloid		Stern & McAfee et al 1966 (84)
"	< 300	Atkins et al 1969 (85)
"	~ 300	Bergqvist et al 1983 (86)
^{51}Cr -Sheep Red Blood Cells		Bradfield 1977 (87)
^{51}Cr -Fixed Sheep Red Blood cells		Schneidkraut & Loegering 1981 (88)
Polyvinyl pyrrolidone (PVP)		Morgan & Soothill 1975 (89)
Liposomes		Hinkle et al 1977 (90)

1.9. Depression of reticuloendothelial function

Interference with normal macrophage function has been implicated as a mechanism by which colloidal thorium dioxide depressed antibody formation (97). Early experimental attempts to produce a functional "blockade" of the RES were based on the concept of the physical saturation of RE cells by inert colloids (98). In studies using colloidal carbon, it was shown that the previous injection of a small dose of India Ink (8 mg carbon/100 g) slowed the rate of clearance of carbon particles when subsequent injections of the same dose were given (72). The intravenous administration of colloidal carbon or Thorotrast (thorium dioxide) to rats was followed by a decreased blood clearance rate of colloidal carbon (99).

The reticuloendothelial depressant effect of methyl palmitate has been thoroughly evaluated (100, 101, 102). Histological evidence indicated that the RE depressant effect was not due to destruction of RE cellular elements but rather to an interference with the phagocytic activity of Kupffer cells (100). The depressed functional state of the RES at the time of antigenic challenge with sheep erythrocytes was associated with a profound inhibition of antibody formation (100). Methyl palmitate did not influence the levels of leucocytes or lymphocytes in the peripheral blood (100). The early tissue distribution of ¹⁴C-labeled methyl palmitate was not comparable to the distribution of colloids known to be phagocytized by the RES (101). RE depression by methyl palmitate was not due to hydrolysis and formation of methanol, since methanol per se did not influence RE function (101). Evidence of saturation of the phagocytic cells with lipid has not been demonstrated following the administration of methyl palmitate (101). Studies by Saba and DiLuzio regarding the possibility of a humoral, opsonic defect induced by methyl palmitate revealed that the phagocytic depression was primarily due to a selective hepatic and splenic macrophage impairment coupled with only a slight reduction in opsonic activity (102).

Numerous other compounds depressing reticuloendothelial phagocytic activity have been described such as dextran sulphate (87, 103), gadolinium chloride (104), colloidal gold (105), and plasma substitutes such as dextran 40 and 70, gelatine (Haemaccel[®]) and polyvinylpyrrolidone (PVP) (106). The in vitro depressant effect of the plasma substitutes were more pronounced in combination with burn trauma, and seemed to be mediated through interaction with humoral factors. A transitory depression of RE function has been observed in rats following surgery. This depression was not associated with alterations in liver blood flow (32). The RE depression in rats after surgery was closely related to a decreased opsonic activity of serum and was followed by a compensatory increase in RE activity 3-4 hours after surgery (107).

1.10. Stimulation of reticuloendothelial function

Administration of endotoxin to animals decreases the phagocytic function of the RES as measured by carbon clearance according to Benacerraf and Sebestyen (76). These investigators, however, showed that the prior administration of a small dose of endotoxin rendered the macrophages resistant to the effect of subsequently given endotoxin. A yeast polysaccharide, zymosan, derived from the cell wall of *Saccaromyces Cerevisiae*, was also found to have stimulatory effects on the macrophage system, increasing the rate of clearance of colloidal carbon (76). Histological observations revealed that zymosan was phagocytized by the RES and that the RE stimulation was accompanied by proliferation of reticuloendothelial elements (108) and with the reversible formation of macrophage containing granulomas in the liver (109). The effects of zymosan on the RES have been explored by several investigators (109, 110, 111, 112, 113, 114).

The active RE stimulatory component of zymosan was identified as glucan, a β 1,3-polyglucose (110, 112). The effects of zymosan in activating the C3 part of the complement has also been observed (115).

Estrogens were also shown to increase RE function in animals (116).

1.11. Lysosomal enzymes and reticuloendothelial function

The ability of mononuclear phagocytes to release different compounds into their pericellular environment has been demonstrated and has been the subject of recent reviews (69, 117, 118). These compounds include hydrolytic enzymes, such as lysosomal acid hydrolases, lysozyme and neutral proteinases; arachidonic acid oxygenation products, such as prostaglandins and thromboxane; and components of the complement system, such as C3a (118).

Intracellular distribution of enzymes in liver macrophages has been studied. Differential centrifugation of whole cell homogenates has shown that acid glucosidase, acid phosphatase, acid protease and lysozyme were largely sedimentable in the lysosomal fraction (119). These studies revealed that elicited (activated) macrophages showed intracellular elevations of most acid hydrolases.

The phagocytosis of digestible and non-digestible particles by macrophages and peripheral leucocytes in vitro was associated with release of lysosomal enzymes (120, 121, 122). The release of β -hexosaminidase (β -N-acetylglucosaminidase, EC 3.2.1.30) during phagocytosis in vitro of opsonized latex particles was more pronounced from monocyte-derived macrophages than from peripheral blood monocytes (123). Musson et al also observed release of β -hexosaminidase by cultured macrophages in response to serum opsonized zymosan. In vivo studies have revealed increased serum activity of lysosomal enzymes during phagocytosis of zymosan or glucan in rats (114, 124). In addition to the secretory

capacity of macrophages vis a vis lysosomal enzymes, recent observations have identified the importance of macrophages in clearing these enzymes from the blood (68, 125, 126).

1.12. Reticuloendothelial function and tumor growth

The suspicion that the RES plays a major role in the host defense against neoplasia dates back to the 19th century. These early studies which were difficult to interpret because of the lack of methods to evaluate RE function have been reviewed by Stern & Willheim (127). With the introduction of the colloid clearance technique experimental tumor growth was found to be associated with enhancement of clearance of injected particles from the blood (128, 129, 130).

In subsequent experiments in mice, Old et al (62) observed that enhanced phagocytic activity, as reflected by the intravascular removal of colloidal carbon, increased during the early phase of sarcoma and carcinoma tumors. Prior to the death of animals, the disappearance rate of colloidal carbon returned to normal (62). An increase in tumor size correlated with a decline of RE function. Studies in humans with gastric cancer revealed a more depressed RE function in patients with locally advanced disease than in patients where radical surgery was possible (131). Other observations have shown an increased RE function, measured by particle blood clearance, in patients with cancer (132, 133).

The contributing role of macrophages in the host defense system has been amply demonstrated. Phagocytosis of tumor cells by macrophages has been reported in vivo and in vitro (65, 134, 135, 136). Birbeck and Carter (137) investigated the role of macrophages in the dissemination of tumor cells. The difference in behavior between two transplantable hamster lymphomas, one of which metastasized while the other only grew at the site of implantation was studied. The non-metastasizing lymphomas contained large macrophages filled with tumor cells in various stages of digestion. The lymphoma that metastasized also contained macrophages, but these were small, unactivated cells with limited phagosomes. The proportion of macrophages in a number of syngeneic mouse and rat fibrosarcomas was observed to range from 4 to 55 % of the total cell population (138). It was also observed that, two weeks after implantation of a tumor cell suspension freed of macrophages, the proportion of macrophages in the growing tumors was equal to controls. Baum and Fischer (139) found that between 4 days and 2 weeks of progressive growth of a syngeneic mammary tumor in mice, there was a significant increased production of macrophage colonies in vitro by bone marrow cells. These macrophages were cytotoxic to cells from the immunizing tumor as long as a tumor was present in the animal from which bone marrow was derived. This effect remained up until 21 days after tumor removal (140). "Armed" monocytes may thus originate from the bone marrow, but their cytotoxic effect could later decrease in spite of remaining tumor growth (139). In

vitro studies have revealed that toxoplasma-activated macrophages were cytotoxic to tumor cells or transformed fibroblasts but not to non-transformed fibroblasts (141, 142). Toxoplasma activated macrophages have been demonstrated to be cytotoxic in vitro to tumor cells, while lymphocytes from either normal or toxoplasma infected mice did not exert this effect (143).

It has been revealed that certain tumors produced substances which abrogated the monocyte chemotherapeutic response (144). Evaluated by bioassay technique, human recognition factor activity (opsonic activity) was decreased in patients with cancer (35). The inability of serum obtained from cancer patients to support phagocytosis was not related to the presence of a phagocytosis-inhibiting factor (145). Experimental studies in rats employing Shay chloroleukemia transplants have demonstrated a marked decrease of humoral recognition factor activity which coincided with the onset of leukemia (34). An ability of tumors to counteract host macrophage resistance to *Listeria monocytogenes* has been demonstrated (146). A soluble factor was isolated from the supernatant of in vitro cultured colonic carcinoma which inhibited macrophage phagocytosis (147).

The administration of different substances which stimulate RE function has been associated with decreased experimental tumor growth. Zymosan or glucan has been used extensively in this context (109, 112, 148, 149, 150, 151, 152).

The influence of RE depression on tumor growth was evaluated by Fischer and Fischer, who found an augmented growth of intrahepatic tumor after portal administration of tumor cells in the rat following administration of colloidal carbone or Thorotrast (99) or after surgical trauma (153). A decreased resistance to intravenous tumor cell challenge during reticuloendothelial depression following surgery was also reported by Saba and Antikatzides (154). The important role of the RES as a major host defense system has been reviewed recently (63, 64, 66, 155).

2. PURPOSE AND AIM OF THE STUDY

Among the many preparations known to be extracted from the blood by the RES, $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid has been used extensively in nuclear medicine for imaging of the liver and spleen. With a method available to prepare a highly standardized $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid (156), it was considered attractive to evaluate its potential as an agent for determination of reticuloendothelial function, adopting the technique of dynamic uptake measurement (I).

Further characterization of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid was considered necessary, especially with reference to the number and the size of the colloid particles.

As the process of phagocytosis could be accompanied by the release of lysosomal enzymes, plasma levels of a lysosomal enzyme, β -hexosaminidase, were determined following injection of colloids or other agents known to interfere with the function of the reticuloendothelial system (III).

In order to evaluate the location of injected substances, morphological studies of liver and spleen were carried out after the administration of RE test colloids (I, III). Histological studies of lung, liver and spleen were performed to evaluate macrophage activation after zymosan injection (III).

The effect of stimulation or depression of the RES on the enzyme levels of β -hexosaminidase were evaluated to determine if β -hexosaminidase levels could serve as markers of reticuloendothelial function (IV). In order to determine if the applied technique could uncover effects on the RES of blocking agents, RE function was measured after administration of different agents known to suppress RE function.

RE function was also evaluated in animals with experimental tumors to study the influence of tumors - including the significance of tumor size and site - on the RES (II). The effect and duration of reticuloendothelial depression, induced by methyl palmitate, on the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid uptake rate and the influence of this RE depression on the growth of an experimental liver tumor were also explored (V). In this study histological examinations of liver and tumor tissue were included.

3. MATERIALS AND METHODS

3.1. Animals

Inbred Wistar rats were used in all experiments. The rats were obtained from the Wallenberg Research Institute, Lund, Sweden. All rats were fed on standard food pellets and water ad libitum. Animals of both sexes, weighing 150-200 g were used for the experiments reported in papers I-IV. For studies on liver tumor growth (V), male rats, 320-370 g, were used.

3.2. Test substance

The test substance, $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid ($^{99}\text{Tc}^{\text{m}}_{27}\text{S}_7$), was prepared from 48 μmol sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) and 9 μmol potassium perrhenate (KReO_4) according to Persson and Naversten (156). The particle size of the colloid was estimated by scanning electron microscopy, with a Coulter Nano-Sizer[®] (Coulter Electronics Ltd) and by ultrafiltration through polycarbonate (Nuclepore[®]) filters (86).

The $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid was prepared within a period of 2-3 hours before the experiment and was tested in each case for the labeling efficiency of the preparation with gel chromatography column scanning technique (157, 158). An example of a typical activity distribution on a Sepharose 4B column for the $^{99}\text{Tc}^{\text{m}}_{27}\text{S}_7$ -colloid is depicted in Figure 2.

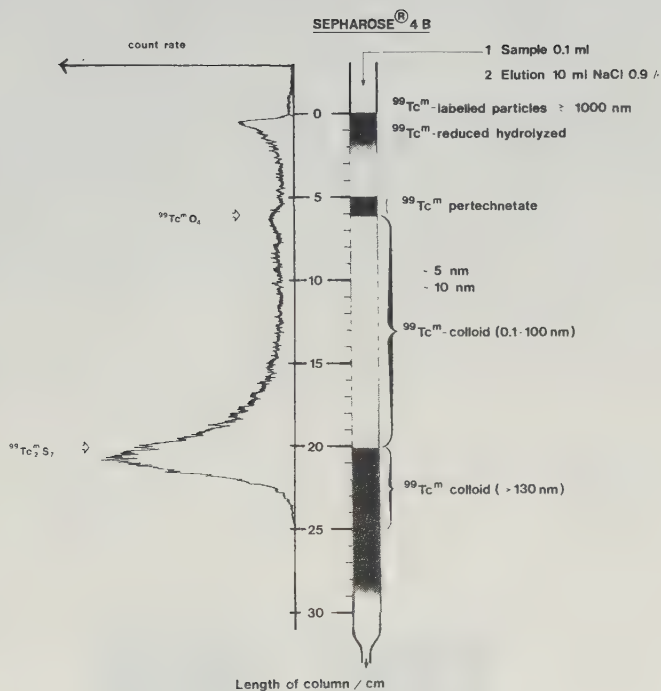


Fig 2. The principle of testing $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid by gel chromatography column scanning. Left, the scanning profile from a $^{99}\text{Tc}^{\text{m}} \text{S}_7$ -colloid preparation. Right, the localization of the activity profiles of different colloid sizes.

3.3. Measurements of RE function

3.3.1. Scintillation camera technique

Under general anesthesia with Nembutal, 6 mg/kg, the right jugular vein was cannulated and a fine catheter was introduced and fixed with its tip in the superior caval vein. With the animal fixed on the scintillation camera, 0.5 ml test colloid suspension was injected rapidly for a period of 4-6 s. Registration of the activity distribution in the animals started immediately at the onset of injection.

In the first paper (I), a scintillation camera (Pho/Gamma III HP, Searle Radiographic Inc) equipped with a 16 000 hole collimator was used. Sequential images were taken every 15 s from the start of injection and continued for 15 min. Data were stored on magnetic tape. Regions of interest were selected over the liver and heart (representing blood activity) and time activity curves were generated. The time activity data were punched on a paper tape and transferred to a computer (Digital Equipment PDP 11/45). A scintillation camera and data aquisition scheme are depicted in Figure 3.

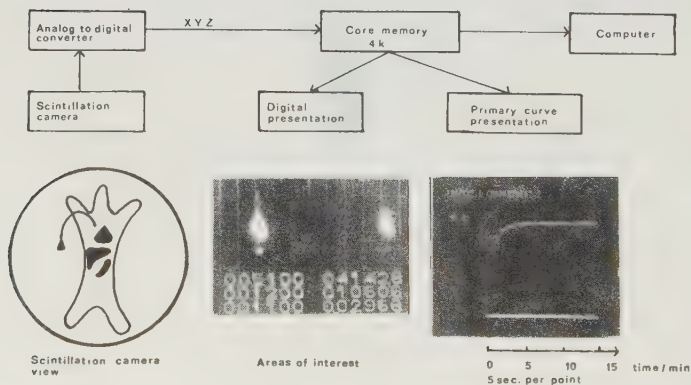


Fig 3. Scintillation camera and data aquisition scheme. Scintigraphic image of liver, spleen and heart of a normal rat. To the right are the corresponding time-activity curve of the liver and the curve fitted according to Equation 9.

For the subsequent studies (II, V), the kinetics of activity distribution were measured with a scintillation camera (Searle, LFOV, Searle Radiographic Inc) equipped with a 39 500 hole collimator. Sequential images were recorded during a 15 min period from the onset of injection. Three frame groups were stored with 16, 12 and 20 frames at 7.5, 15 and 30 s intervals, respectively. Regions of interest were selected from the scintigraphic image over the liver and the head (representing blood activity). Time activity curves were generated and evaluated with a mini-computer system (Digital Equipment Gammall).

3.3.2. Computer analysis

The final percentage activity uptake in the liver can be calculated or measured in different ways, the easiest being extirpation of the liver and measurement of the organ activity in e.g. an ionization chamber or NaI (Tl) well counter (III) or by placing the organ on the scintillation camera (II).

The activity in the liver can also be calculated from the stored scintillation camera images by using the calibration factor, α , i.e. the count rate per activity unit in the liver region (I). This factor can be obtained by having a known amount of $^{99}\text{Tc}^{\text{m}}$ in a rat liver phantom placed in a rat phantom. The percentage activity uptake in the liver, $P(t)$, can then be expressed by Equation 5.

$$P(t) = N(t) \cdot \{\alpha \cdot \Delta t \cdot A(0)\}^{-1} \cdot \exp\{\lambda \cdot t\} \cdot 100 \quad (5)$$

$N(t)$ is the net number of counts in the liver region at time t , Δt the frame time, $^{99}\text{Tc}^{\text{m}} A(0)$ the injected activity and λ the physical decay constant of $^{99}\text{Tc}^{\text{m}}$.

To estimate the flow rate of the labeled colloid into the liver, k_1 , and into the extrahepatic RES, k_2 , an open, two-compartment model was used, schematically presented in Figure 4.

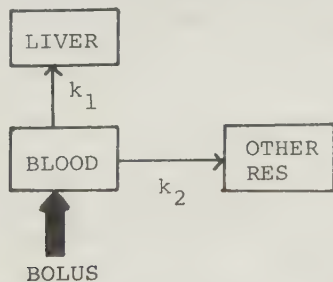


Fig 4. Schematic presentation of the compartment analysis for calculation of the colloid uptake rates k_1 and k_2 .

Using the symbols in the figure, the rate of change of the colloid in the blood (B) and in the liver (L) with time could be expressed:

$$\frac{dB}{dt} = -B(k_1 + k_2) \quad (6)$$

$$\frac{dL}{dt} = B \cdot k_1 \quad (7)$$

The percentage amount of activity in the blood and the liver at any time (t) can then be expressed by the Equations 8 and 9.

$$B(t) = 100 \cdot e^{-(k_1+k_2+\lambda)t} \quad (8)$$

$$L(t) = 100 \cdot \frac{k_1}{k_1+k_2} \cdot e^{-\lambda t} \{1 - e^{-(k_1+k_2)t}\} \quad (9)$$

The rate constants k_1 and k_2 were obtained by fitting these equations to the experimental curves for each animal with a non-linear least-square fit (159).

The uptake of colloids by the RES was mostly described either by the phagocytic index (k_{phag}) or by the half-time of clearance from the blood, $t_{1/2}$, according to Equations 1 and 3. To compare the results from the two-compartment analysis to calculations of phagocytic index, this index was calculated in the first paper (I). As the first very rapid component of the liver uptake curve (40-60 s after injection) represented both mixing of the test substance with the blood and phagocytosis by the liver, calculations of k_{phag} were made in the second part of the curve representing mainly phagocytosis of the colloid. Blood mixture of the colloid could then be assumed to be complete. The phagocytic index was calculated in this second part of the curve using the formula:

$$k_{phag} = \log \frac{A(120)}{A(60)} \quad (10)$$

where $A(120)$ was the uptake of activity (i.e., proportional to the number of particles) in the liver at 120 sec after injection, and $A(60)$ was the uptake at 60 sec.

The k_{phag} values were also determined from the compartment analysis (k_{phagCM}) by putting the obtained k_1 and k_2 values into Equation 9 and

thus the activity in the liver could be determined at any time after injection.

The accumulated activities in the liver and spleen during 15 min of registration were evaluated separately. After completion of the uptake study the animals were sacrificed and the accumulated activities in the extirpated liver and spleen were determined and compared with the organ activity in the intact animal.

3.4. Tumors

The experimental tumor studied was a N-methyl-N'-Nitrosoguanidine-induced adenocarcinoma of the colon (NGW), obtained from the Wallenberg Research Laboratory in Lund. The tumor was propagated by intra-renal transplantation in inbred Wistar rats. Under sterile conditions, a tumor cell suspension was manufactured using trypsin; vital cell counts were made in a hemocytometer by adding trypan blue. Tumor transplantation to the liver (II, V) and to the dorsal subcutaneous tissue (II) was performed with 1.0×10^6 cells and to the kidney (II) with 0.5×10^6 cells. Inoculation to the liver was performed into the periphery of the central lobe, on the anterior surface, just beneath the liver capsule. Access to the liver and the kidney was gained through a midline abdominal incision with the animal under ether anesthesia. Measurements of tumor size were performed with vernier calipers in the greatest (a) and smallest (b) superficial diameters on the ventral side of the liver lobe. Tumor volume V (mm^3) was approximated according to Equation 11 and transformed to logarithmic values.

$$V = \frac{a \cdot b^2}{2} \quad (11)$$

A material presentation of the study on reticuloendothelial function and tumor growth is given in Table III.

Table III. Material presentation from study II.

Group	Number	Number tumor cells inoculated $\times 10^6$	Time interval (days)	Tumor weight (gram)	
				Range	Mean
I. Control	12				
II. Liver tumor	8	1.0	18-21	0.8-2.3	1.6
III. Retroperitoneal kidney tumors	5	0.5	24-28	2.9-20.7	7.0
IV. Subcutaneous tumors	8	1.0	30-36	0.3-2.8	1.3

3.5. Morphological studies

Liver specimens were collected from 8 rats, killed 1, 3, 5, 10, 30, 60 min and 24 and 48 h after administration of the sulphur colloid (I) or the ¹⁹⁸Au colloid (III). For light microscopy, slices were fixed in 4 % formaldehyde, embedded in paraffin and stained with hematoxylin, erythrosine, van Gieson, the Turnbull-blue reaction for iron and with Fouchet's reagent for bilirubin pigment (I, III).

Twenty-eight rats were used for histological examination of the lung, the liver and the spleen after zymosan injection. Under ether anesthesia 16 rats received an intravenous injection of zymosan (3 mg/100 g) and 12 rats were injected with an equal amount of saline. All injections were made through a jugular catheter. Four zymosan-injected animals and 3 control animals were sacrificed at each of the following intervals after the injections: 20, 60 and 120 min and 24 h. Specimens from lung, liver and spleen were collected, prepared as described above, and stained with hematoxylin-erythrosine, PAS and PAS preceded by diastase treatment.

For light microscopy studies after methyl palmitate administration, specimens from lung, liver, and spleen were collected 1 and 4 days after administration of methyl palmitate (V). In the study of liver tumor growth (V), specimens of the tumor and surrounding liver parenchyma were collected at 7, 14 and 18 days after tumor inoculation. These specimens were stained with hematoxylin-erythrosine, the periodic acid-Schiff reaction and the Turnbull blue-method for iron. Unembedded slices were stained with scarlet red for fat. The extent of necrosis, fat and iron pigment was evaluated from - to +++ (Table XVI).

For electron microscopy (I), liver slices were fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer and 0.1 M sucrose, pH 7.2 at +4°C for 4 h with post-fixation in 2 % O_3 in 0.1 M s-collidine buffer, pH 7.4 at +4°C for 1 h. The slices were embedded in Epon and 1 μ m sections were stained with methylene-blue thionin. Ultrathin sections were contrasted with uranyl acetate-lead citrate and examined in a Zeiss EM 10 electron microscope.

3.6. Preparation of other agents

Methyl palmitate (Sigma Co) was prepared as an emulsion by sonification for 10 min 5 % dextrose solution and 0.1 % Tween 20 (100). The concentration of methyl palmitate in the emulsion was 400 mg/ml. Sterilization was performed by boiling in a water bath for 1 h. The administered dose was 200 mg/100 g, given intravenously once (III) and on two consecutive days (IV, V).

Zymosan (Sigma Co) was suspended in 0.9 % saline at a concentration of 10 mg/ml, and sterilized in boiling water for 1 h (114). Zymosan was

administered intravenously in a dose of 3 mg/100 g once (III) and on two consecutive days (IV).

Standard preparations of colloidal gold, ^{198}Au (Amersham Radiopharmaceuticals) (III) and gelatine, Haemaccel[®] (Behring) were employed (I, III). The design of the studies concerning β -hexosaminidase is given in Tables IV (III) and V (IV).

Table IV. Design of study III.

Groups	Administered agent	Concentration	Amount
A	Saline (control)	0.9 %	2 ml
B	$^{99}\text{Tc}^{\text{m}}$ -sulphur colloid	12 mg $\text{Na}_2\text{S}_2\text{O}_3$	1 ml *
C	^{198}Au -colloid		1 ml *
D	Gelatine (Haemaccel [®])	3.5 %	2 ml
E	Alcohol	96 %	0.25 ml *
F	Methyl palmitate	400 mg/ml	200 mg/100 g *
G	Zymosan	10 mg/ml	3 mg/100 g *

* Diluted with saline to a volume of 2 ml.

Table V. Design of study IV.

Group	Number	Days 1 and 2		Day 3			
		Agent 1	Dose	Agent 2	Dose	Agent 3	Dose
A	5	-	-	-	-	Zymosan	3 mg/100 g
B	5	Zymosan	3 mg/100 g	-	-	Zymosan	3 mg/100 g
C	5	Methyl palmitate	200 mg/100 g	-	-	Zymosan	3 mg/100 g
D	5	Zymosan	3 mg/100 g	Alcohol 96 %	0.25 ml	Zymosan	3 mg/100 g
E	5	-	-	Alcohol 96 %	0.25 ml	Zymosan	3 mg/100 g

The influence of alcohol on the release of β -hexosaminidase after zymosan injection was studied (IV). Alcohol was administered as 0.25 ml 96 % alcohol in 2 ml saline. Provided the blood volume of the rat was 8 ml/100 g, the calculated blood concentration of alcohol could be about 1.3 o/oo.

3.7. Enzyme analysis

For enzyme studies, heparin-plasma samples (0.5 ml) were collected from the jugular vein catheter (III, IV) or from the retroorbital plexus (V). All samples were analyzed for plasma β -hexosaminidase by a previously described method (160). The coefficient of variation for the method was below 10 %. Plasma samples were analyzed for lactate dehydrogenase (LD, E.C. 1.1.1.27) according to a method described by Keiding et al (161).

3.8. Statistical analysis

Analysis of k_1 and k_2 values from the compartment analysis was done by computing single best-fit linear relationship between k_1 and k_2 by a technique described by Barlett (162).

When comparing differences, two-tailed Student's t-test was used. The significance was also verified with the non-parametric Wilcoxon rank sum test. Differences in survival (V) were tested according to the χ^2 test. A p value of less than 0.05 was considered significant.

The labeling efficiency of the colloid was observed to range between 97 % and 100 %. By increasing the amount of sodium thiosulphate the labeling efficiency, however, decreased and was only 60 % for a preparation containing 300 mg $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$. Studies of the liver uptake of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid prepared with different amounts of sodium thiosulphate revealed that k_{phag} decreased with increasing amounts of $\text{Na}_2\text{S}_2\text{O}_3$ (Fig 5).

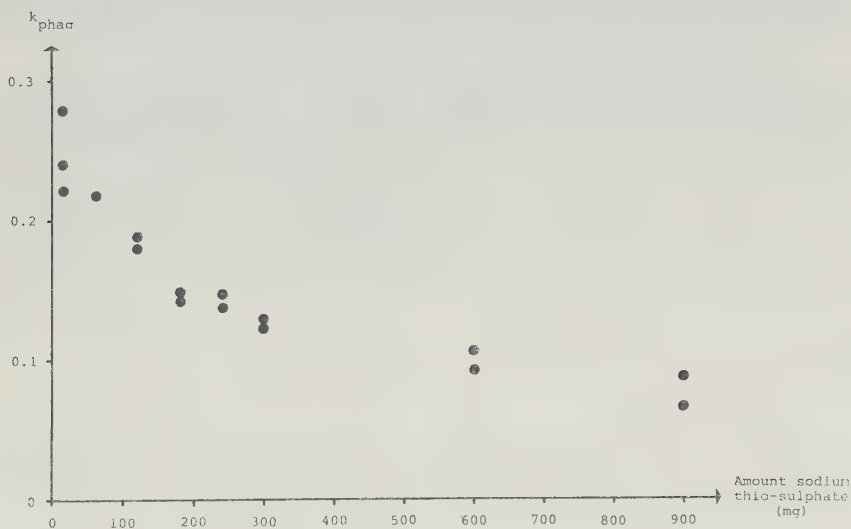


Fig 5. Phagocytic index k_{phag} in relation to the amount of sodium-thio sulphate in the $^{99}\text{Tc}^{\text{m}}\text{S}_7$ colloid preparation.

By estimation of particle size with Coulter Nano-Sizer[®], the mean value for the standard $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid (containing 12 mg $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) was 390 nm. For a preparation containing 300 mg sodium thiosulphate the mean particle diameter was 230 nm.

Particle size measurements at different intervals from the preparation of the colloid suspension showed an increase of mean particle diameter with time. Mean particle diameter was 390 nm 0-3 h, 410 nm 3-6 h and 750 nm 6-9 h after colloid preparation. After incubation with normal rat plasma at 37°C for 1 h, the mean particle diameter increased about 100 nm. The "polydispersity index" which was an index of the size range, also increased with time.

The uptake rates of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid, as measured by the values of k_1 (liver) and k_2 (extrahepatic RES) from the two-compartment analysis for 10 normal rats and 5 rats pretreated with Haemacel[®] are depicted in Table VI. Haemacel[®] was given in a dose of 1-3 ml 10 min prior to registration (I).

Table VI. k_1 and k_2 values for normal and Haemacel[®]-treated rats (mean $\pm$ SD). p values for comparison according to the two-tailed Student's t-test.

	Normal n = 10	Haemacel n = 5	p
$k_1 \cdot 10^{-1}$	(0.23 $\pm$ 0.03)	(0.18 $\pm$ 0.04)	p < 0.01
$k_2 \cdot 10^{-2}$	(0.30 $\pm$ 0.05)	(0.35 $\pm$ 0.05)	p < 0.05

A significant reduction (p < 0.025) in the accumulated activity in the liver was observed for Haemacel[®] treated rats compared to controls (Table VII).

Table VII. Total liver uptake of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid in normal and gelatin pretreated rats, in percentage of the injected colloid dose (I).

	n	% Activity Range	Mean	
Normal	10	84.7-97.2	91.2	
Haemacel [®]	5	78.1-90.7	83.7	p < 0.025

Statistical analysis for 95 % confidence by Student's t-test

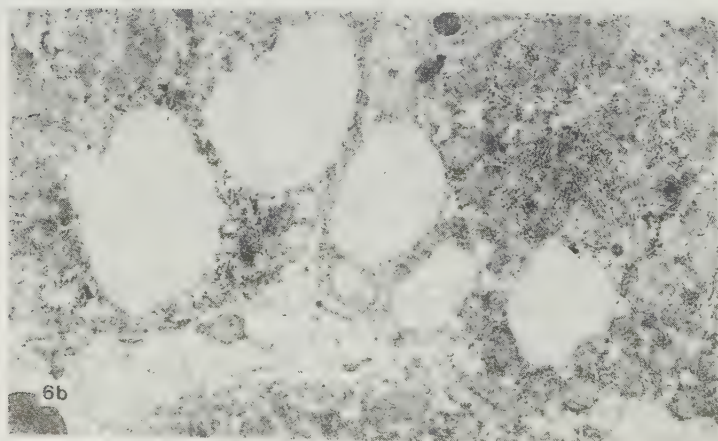
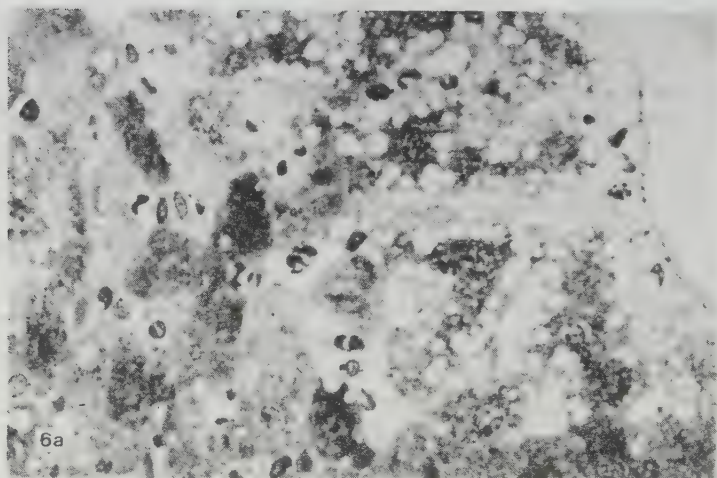
The k_{phag} , k_{phagCM} and k_1 values for normal rats are given in Table VIII. Comparison of k_{phag} to k_{phagCM} revealed no significant difference in the range of values and the standard deviation was the same. k_1 values were, however, more narrowly distributed with less deviation from the mean value.

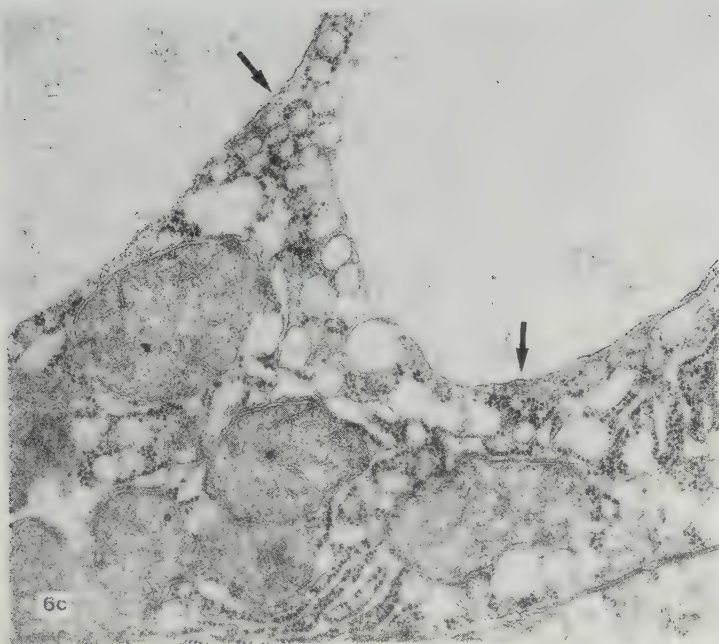
Table VIII. Comparison of different methods for estimation of liver phagocytosis in normal rats:

	$\bar{x}$	Range	SD	
k_{phag}	0.18	0.11-0.24	0.04	22 %
k_{phagCM}	0.21	0.15-0.32	0.05	23 %
$k_1 \cdot 10^{-1}$	0.23	(0.19-0.29)	0.03	13 %

Light microscopy studies showed vacuoles in hepatocytes especially in the central part of the lobuli, from 1 min to 24 h, most pronounced at 3-30 min after $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid injection. The vacuoles were often found in rows in the periphery of the cells in close to the sinusoids (Fig 6a). In electron microscopy the vacuoles were often found to be surrounded by a membrane and to contain a flocky substance (Fig 6b, c). No pathological changes were noted in other parts of the cells. Vacuoles were also found in large amounts in the sinusoidal cells.

Fig 6 A-C. A. Light microscopical section. Rat killed 10 min after administration of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid (I). Vacuoles are seen in the cytoplasm of the parenchymal cells, especially in the zone close to the sinusoids, in the central parts of the lobuli. Thick section of Epon-embedded specimen. Methylene blue-thionin x 600. B, C. Electron microscopical sections demonstrating vacuoles in the periphery of the parenchymal cells close to the sinusoids (B. x 6 700). The vacuoles are often delimited by a membrane (arrows). The mitochondria and the rough-surfaced endoplasmic reticulum in the lower part of the picture are well preserved (C. x 40 000). Uranyl acetate-lead citrate.





Light microscopical examination after ^{198}Au colloid administration (III) demonstrated vacuoles in the hepatocytes and sinusoidal cells from 1 to 60 min after injection. No changes were detected after 24 and 48 h. Examination of the spleens revealed no changes at any time.

Histological examination of lung, liver and spleen after different intervals following zymosan injection revealed no changes in the spleens. In the livers the sinusoidal cells in the peripheral parts of the lobuli were larger and contained more PAS-positive, diastase-resistant material at 1 and 2 h than at 20 min and 24 h. At all times there was very little of such material in the controls. In the areas with prominent sinusoidal cells, globules with this staining characteristic were also found in the liver parenchymal cells and considerably more than at 20 min and 24 h (Fig 7 c). There were almost no such globules in the controls.

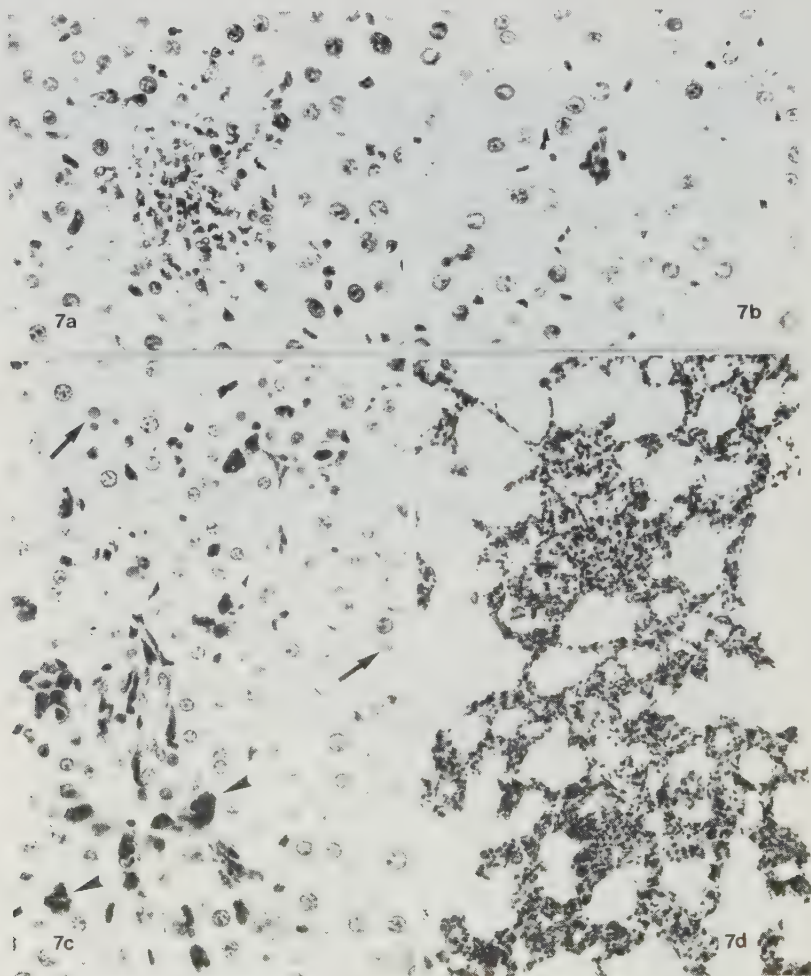


Fig 7. a) Large granuloma in the liver containing both granulocytes and Kupffer-like cells 24 h after zymosan injection. Hematoxylin-erythrosine x 400. b) Small granuloma of probably Kupffer cells in the liver 2 h after administration of zymosan. Hematoxylin-erythrosine x 400. c) Prominent Kupffer cells (Δ -arrow) in the peripheral part of the liver lobulus 2 h after zymosan injection. Globules of varying size are found in several parenchymal cells (arrows). PAS-staining after treatment with diastase. x 165. d) Section of lung 1 h after administration of zymosan. Granulocytes are often found in large amounts in the tissue. Hematoxylin-erythrosine x 165.

Small granulomas of sinusoidal cells were found at 2 h (Fig 7 b). At 24 h there were several granulomas, containing neutrophil polymorphonuclear leukocytes and sinusoidal cells in varying proportions, especially in the peripheral parts of the lobuli and also bordering to the portal tract (Fig 7 a). There may have been necrosis of parenchymal cells in these granulomas. There were no granulomas in the controls.

In the lungs a conspicuous amount of neutrophils were found in the interstitial tissue and/or in the small blood vessels at 20 min and 1 and 2 h often in small heaps (Fig 7 d). There were a few granuloma-like structures at 2 h (Fig 8 a). At 24 h the inner layer of the small blood vessels were often considerably thickened by cells, and neutrophils were often found around the vessels and in larger number than in the control (Fig 8 b). A few granuloma-like areas were also encountered.

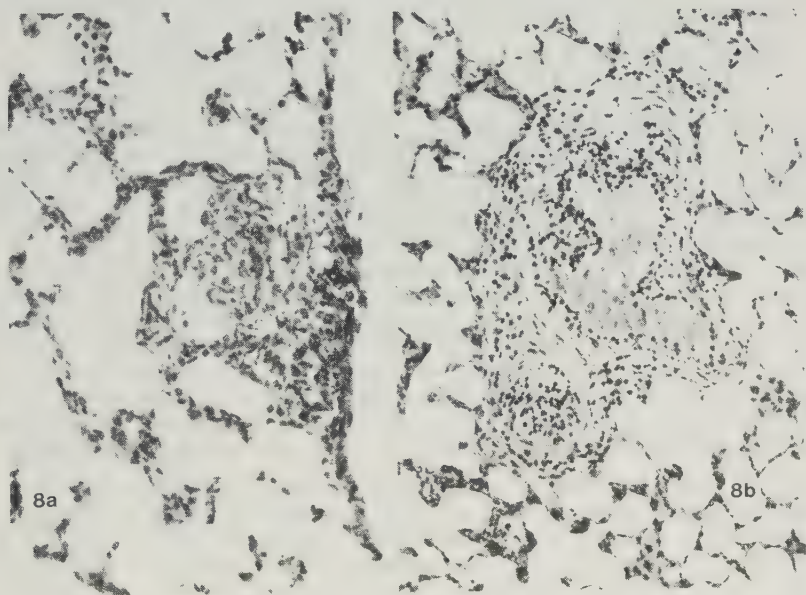


Fig 8. a) A granuloma in the peripheral part of the lung 24 h after zymosan injection. Hematoxylin-erythrosine x 250. b) Blood vessel thickened intima in the lung 24 h after administration of zymosan. Granulocytes are found perivascularly. Hematoxylin-erythrosine x 165.

The distribution of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and the ^{198}Au colloid in various organs and tissues in percentage of injected activity per g is depicted in Table IX (III). Significant differences were registered regarding the uptake of the two colloids in the spleen, lung and bone marrow.

Table IX. Activity distribution in per cent of injected activity per gram tissue (mean $\pm$ SD) in different organs for $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and ^{198}Au colloid. p values for comparison of differences according to Student's t-test.

	Blood	Liver	Spleen	Lung	Bone marrow	Kidney	Muscle
$^{99}\text{Tc}_2\text{S}_7$	0.066 $\pm$ 0.051	13.4 $\pm$ 1.6	6.5 $\pm$ 1.1	0.47 $\pm$ 0.24	0.18 $\pm$ 0.04	0.055 $\pm$ 0.005	0.0018 $\pm$ 0.0004
^{198}Au	0.15 $\pm$ 0.07	14.2 $\pm$ 2.0	3.6 $\pm$ 0.4	0.11 $\pm$ 0.02	2.8 $\pm$ 0.5	0.047 $\pm$ 0.014	0.0028 $\pm$ 0.0009
p	n.s.	n.s.	< 0.001	< 0.02	< 0.001	n.s.	n.s.

In the study of reticuloendothelial function in relation to tumor growth (II), the site and localization of the tumors are presented in Table III. Tumor take in the liver and kidney was 100 % and subcutaneously 80 %. Tumor growth was considerably faster in the liver and kidney compared to the subcutaneous localization. At the time of examination (Table III), all liver tumors were solitary, confined to the inoculated lobe. The subcutaneous tumors were also solitary and well defined. In these groups, no signs of metastatic tumor spread were noted at examination of the animals after RE function tests. In the group in which tumor cells were inoculated into the kidney, all animals revealed extrarenal tumor growth at the time of examination. Retroperitoneal growth was manifested either as per continuitatem spread and/or as lymph node metastases. None of these animals manifested tumor invasion of the liver or signs of distant metastases.

Animals with subcutaneous tumors gained weight during the period from inoculation to examination to the same extent as controls. During the same period, animals with liver tumors manifested weight loss of 5 % and animals with retroperitoneal tumor lost 10 % weight. The relative weights of the liver and spleen (as percentage of total body weight) are given in Table X.

All tumor animals demonstrated a statistically significant increase in the relative weight of the spleen; however, no changes in relative weight of the liver were noted in any of the groups. The uptake rates of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid of the liver, k_1 , and of the spleen (extrahepatic RES), k_2 , are depicted in Table XI together with the accumulated activity in the liver and spleen.

Table X. Relative weights of liver and spleen (percentage of total body weight) and p values for comparison with controls (II).

Groups	Liver		Spleen	
	Relative weight (%)		Relative weight (%)	
	Mean $\pm$ SD	p <	Mean $\pm$ SD	p <
I. Controls	3.6 $\pm$ 0.7		0.26 $\pm$ 0.06	
II. Liver tumors	3.7 $\pm$ 0.3	n.s.	1.10 $\pm$ 0.06	0.001
III. Retroperitoneal tumors	3.4 $\pm$ 0.5	n.s.	0.76 $\pm$ 0.40	0.001
IV. Subcutaneous tumors	3.6 $\pm$ 0.5	n.s.	0.60 $\pm$ 0.09	0.001

Table XI. ^{99m}Tc -sulphur colloid uptake rate and total organ uptake in percentage of injected activity (mean $\pm$ SD). p values for comparisons with controls (II).

Groups	Colloid uptake rate (s^{-1})				Total organ uptake (%)			
	Liver $k_1 \cdot 10^{-1}$	p <	Spleen $k_2 \cdot 10^{-2}$	p <	Liver	p <	Spleen	p <
I. Controls	0.16 $\pm$ 0.02		0.22 $\pm$ 0.07		89 $\pm$ 3		3.2 $\pm$ 1.5	
II. Liver tumors	0.19 $\pm$ 0.01	0.005	0.35 $\pm$ 0.09	0.005	84 $\pm$ 3	0.005	6.4 $\pm$ 1.5	0.001
III. Retroperitoneal tumors	0.12 $\pm$ 0.02	0.005	0.24 $\pm$ 0.08	n.s.	84 $\pm$ 3	0.01	7.6 $\pm$ 2.1	0.02
IV. Subcutaneous tumors	0.18 $\pm$ 0.03	0.05	0.45 $\pm$ 0.17	0.005	81 $\pm$ 8	0.01	5.7 $\pm$ 1.9	0.005

Animals with liver and subcutaneous tumors revealed a statistically significant increase of k_1 and k_2 compared to controls. The total accumulated activity of the liver was decreased in those groups whereas the accumulated activity of the spleen was increased. In the group of animals with larger, retroperitoneal tumors the colloid uptake rate of the liver, k_1 was significantly decreased compared to controls, while the uptake rate of the spleen, k_2 , was unaltered. Compared to controls, the total accumulated activity was decreased in the liver but increased in the spleen.

The release of a lysosomal enzyme β -hexosaminidase (β -N-acetyl glucoseaminidase, EC3.2.1.30) was studied after a single administration of different agents, depicted in Table IV (III). The changes of enzyme activity in the different groups compared to basal enzyme levels at different intervals after injections are given in Table XII.

Table XII. Relative activity $\pm$ SEM of β -hexosaminidase 10, 30 and 60 min after injection of test substances. p values for comparison with basal levels with Student's t-test, *: $p < 0.05$, ***: $p < 0.001$ (III).

Groups	Administered agent	Animals (n)	Basal	10 min	30 min	60 min
A	Control (NaCl)	8	1.00 $\pm$ 0.07	1.04 $\pm$ 0.05	0.86 $\pm$ 0.03	0.89 $\pm$ 0.06
B	$^{99}\text{Tc}^m$ -sulphur colloid	5	1.00 $\pm$ 0.08	1.09 $\pm$ 0.06	0.97 $\pm$ 0.07	0.88 $\pm$ 0.06
C	^{198}Au -colloid	5	1.00 $\pm$ 0.08	0.74 $\pm$ 0.19	0.83 $\pm$ 0.20	0.84 $\pm$ 0.08
D	Gelatine (Haemaccel [®])	5	1.00 $\pm$ 0.08	0.86 $\pm$ 0.05	1.01 $\pm$ 0.10	1.05 $\pm$ 0.03
E	Alcohol	6	1.00 $\pm$ 0.27	0.92 $\pm$ 0.19	1.08 $\pm$ 0.13	1.06 $\pm$ 0.11
F	Methyl palmitate	7	1.00 $\pm$ 0.12	0.92 $\pm$ 0.08	1.02 $\pm$ 0.11	1.10 $\pm$ 0.14
G	Zymosan	5	1.00 $\pm$ 0.09	1.36 $\pm$ 0.07*	1.35 $\pm$ 0.06*	1.63 $\pm$ 0.05***

After zymosan injection the levels of β -hexosaminidase were significantly raised over basal levels at 10 and 30 min ($p < 0.05$) and at 60 min ($p < 0.001$). No difference in enzyme activity was observed between 10 and 30 min; however, a statistically significant ($p < 0.025$) increase was observed between 30 and 60 min. Twenty-four and 48 h after zymosan injection, the β -hexosaminidase activity had returned to basal levels.

The change in β -hexosaminidase activity in zymosan injected rats compared to controls is depicted in Figure 9.

Change in plasma β -hexosaminidase %

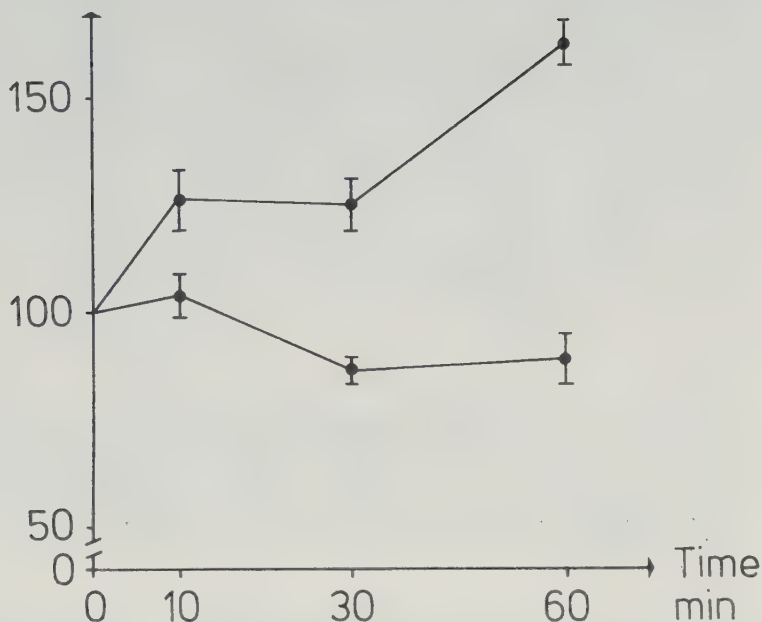


Fig 9. Change in β -hexosaminidase activity (mean $\pm$ SEM) after zymosan injection (upper curve) compared to control (lower curve).

Compared to controls, enzyme activity was higher at 10 min ($p < 0.05$), 30 and 60 min ($p < 0.001$) in zymosan injected animals. For all other agents tested (III), no statistically significant variation in β -hexosaminidase activity was found when compared with basal levels in each group or when compared with controls at corresponding intervals after injection (Table XII).

The plasma levels of β -hexosaminidase for the different groups (IV) at different intervals after zymosan injection are given in Table XIII.

Table XIII. Activity of β -hexosaminidase in serum before (basal) and after zymosan injection. Statistical significance for comparison with basal activity in each group with Student's t-test, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Group	β -hexosaminidase activity					
	Basal	Minutes after zymosan injection				
		10	30	60	90	120
A	12.0 $\pm$ 0.6	12.7 $\pm$ 0.7	17.7 $\pm$ 0.6*	18.1 $\pm$ 0.7***	16.7 $\pm$ 1.2**	17.0 $\pm$ 0.4**
B	11.5 $\pm$ 0.7	14.5 $\pm$ 0.6**	18.7 $\pm$ 1.2**	21.1 $\pm$ 2.2**	21.7 $\pm$ 2.4**	18.7 $\pm$ 1.3**
C	11.4 $\pm$ 0.7	12.4 $\pm$ 1.4	15.3 $\pm$ 0.4*	15.7 $\pm$ 1.1*	16.4 $\pm$ 1.2**	15.3 $\pm$ 1.3**
D	11.8 $\pm$ 0.2	15.8 $\pm$ 1.3*	19.2 $\pm$ 2.0*	18.9 $\pm$ 2.1*	18.1 $\pm$ 1.3**	19.1 $\pm$ 1.8*
E	12.9 $\pm$ 1.0	15.2 $\pm$ 0.5*	19.7 $\pm$ 1.0**	22.9 $\pm$ 1.6**	22.6 $\pm$ 1.4**	20.5 $\pm$ 1.6**

A statistically significant increase of enzyme levels was observed in all groups. Animals pretreated with zymosan or methyl palmitate revealed no deviation in basal levels of β -hexosaminidase when compared to non-pretreated animals. Plasma β -hexosaminidase activity after zymosan injection into rats pretreated with zymosan (B) and methyl palmitate (C) and in non-pretreated rats (A) is depicted in Figure 10.

After 60 min, the level of β -hexosaminidase was significantly higher in zymosan pretreated rats (B) when compared to methyl palmitate pretreated rats (C), with a p value of 0.05. After 90 min, the enzyme level in group B was significantly higher than the enzyme levels of either of the other groups (C and A), with a p value of 0.05 in both comparisons. Alcohol (D and E) did not significantly influence the rise of β -hexosaminidase activity after zymosan injection when compared with the corresponding groups where alcohol was not administered (B and A).

Betahexos-
aminidase
(U/l)

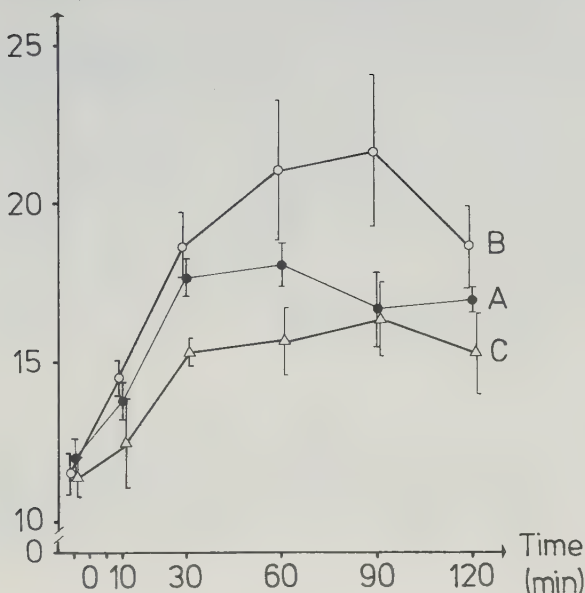


Fig 10. β -hexosaminidase activity after zymosan injection into control animals (A) and into animals pretreated with zymosan (B) and methyl palmitate (C).

The effect on RE function, as measured by the uptake rate of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid, of treatment for two consecutive days with methyl palmitate is presented in Table XIV (V).

When examined on day 3 (methyl palmitate given on days 1 and 2), a pronounced decrease of hepatic colloid uptake rate, k_1 , was observed. At this time no significant effect on extrahepatic colloid uptake rate, k_2 , was noted. Examinations on day 6 revealed no effect on either k_1 or k_2 . The administration of Tween 20 in dextrose for two consecutive days (1 and 2) did not influence k_1 and k_2 values when examined on day 3.

Table XIV. Uptake rate of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid in the liver (k_1) and the extrahepatic RES (k_2). p values compared with control (A).

Groups	n	Colloid uptake rate (s^{-1})			
		Liver		Extrahepatic RES	
		$k_1 \cdot 10^{-1}$	p	$k_2 \cdot 10^{-2}$	p
A. Control, no treatment	5	0.18 ± 0.01		0.26 ± 0.02	
B. Tween 20 + dextrose	5	0.18 ± 0.01	n.s.	0.23 ± 0.05	n.s.
C. Methyl palmitate 3 d	5	0.062 ± 0.051	< 0.001	0.18 ± 0.06	n.s.
D. Methyl palmitate 6 d	5	0.20 ± 0.02	n.s.	0.23 ± 0.11	n.s.

n.s.: not significant at the 0.05 level

All animals developed liver tumors. No mortality was registered in the control group until day 18. However, in the methyl palmitate treated group only 4 of 14 survived until day 18. Survival in control animals was 19 ± 2 days (mean $\pm$ SD) and, in methyl palmitate treated animals, 15 ± 3 days. The difference was statistically significant with a p value of < 0.001 (Fig 11).

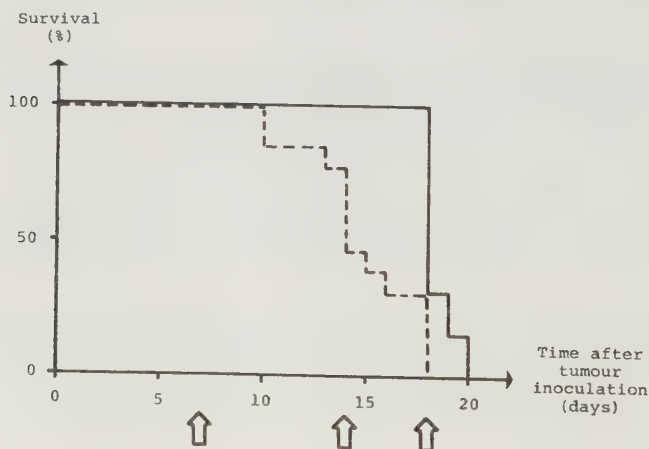


Fig 11. Survival curves for control animals (solid line) and for methyl palmitate treated rats (dotted line). Arrows indicate days of tumor examination.

A significant weight loss was observed in both groups during the first week after tumor inoculation; it was more pronounced in methyl palmitate treated animals (Fig 12).

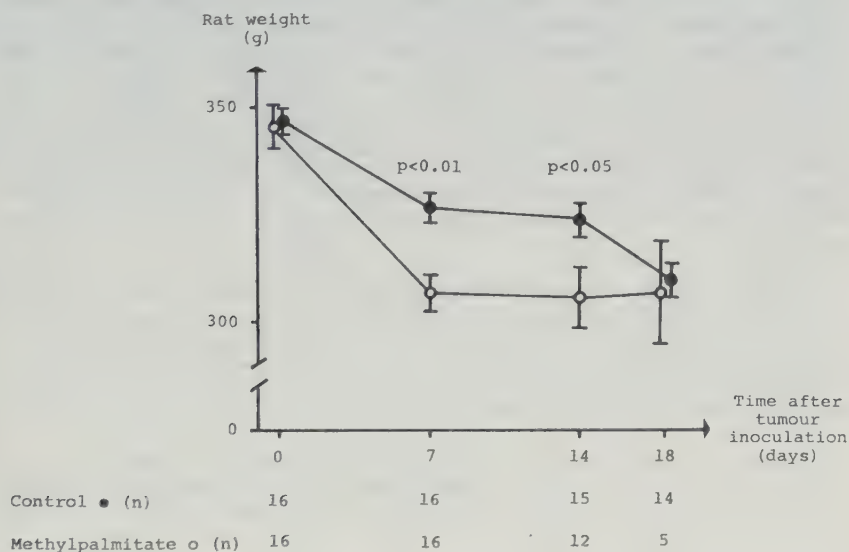


Fig 12. Weight of rats (mean $\pm$ SEM) during liver tumor growth. p values for comparison of differences by Student's t-test.

In the methyl palmitate treated group no further weight loss was noted but control animals lost weight between day 14 and day 18.

Table XV. Log tumor volume (mm^3) (mean $\pm$ SD) for measurements at 7, 14 and 18 days after tumor inoculation. p values for difference according to Student's t-test.

	7 days		14 days		18 days	
	n	volume	n	volume	n	volume
Control	16	2.12 $\pm$ 0.20	15	2.97 $\pm$ 0.24	14	3.82 $\pm$ 0.20
Methyl palmitate	16	2.82 $\pm$ 0.27	12	3.54 $\pm$ 0.29	5	3.83 $\pm$ 0.05
		p < 0.001		p < 0.001		p n.s.

Table XV shows the logarithms of liver tumor volumes for different times. A significant difference was noted in tumor size at 7 and 14 days, with the methyl palmitate treated rats having larger tumors. At day 18, no significant difference in liver tumor size was noted. After 7 days 2 of 16 control animals and 6 of 16 methyl palmitate treated rats had signs of minor extrahepatic tumor growth confined to the abdominal wall. No animals showed signs of ascites or cachexia at this time. After 14 days all animals had signs of extrahepatic tumor growth. In the control group this was confined to the abdominal wall and none of these had ascites or showed signs of malnourishment. All 12 surviving methyl palmitate treated rats appeared malnourished with shaggy fur. At laparotomy all these revealed significant extrahepatic tumor growth with peritoneal carcinomatosis and ascites. At day 18, all rats had developed these signs of extensive intraabdominal tumor growth.

The enzyme levels of both β -hexosaminidase and LD revealed no change either after methyl palmitate or after dextrose administration, and no statistically significant changes in enzyme activity were noted during the course of the experiment. It was not possible to detect any differences in the levels of β -hexosaminidase or LD between the two groups of animals during tumor growth.

Histological examinations after methyl palmitate administration revealed no signs of particle phagocytosis or microembolization in the lungs. A slight increase of fatty vacuolization in the liver parenchyma cells was detected at day 3. One rat examined on day 6 (3 days after methyl palmitate administration) showed increased iron deposition in the phagocytes of the liver. No signs of liver necrosis were seen after methyl palmitate administration. The results of the histological examinations of the tumor and surrounding liver parenchyma are depicted in Table XVI. Iron deposits in the phagocytes of the liver were present to the same extent in both groups. Lipid vacuolization and necrosis in the parenchymal cells of the liver were absent at day 7 in both groups but pronounced at day 14 in the methyl palmitate treated animals. At day 18 no differences were noted. Tumor necrosis was observed to a similar extent in both groups and increased with time.

Table XVI. Histological evaluation of tumor and surrounding liver parenchyma 7, 14 and 18 days after liver tumor inoculation. Grading from - to +++, see text.

Days after tumor ino- culation	LIVER				TUMOR			
	Iron pigmentation in phagocytes		Lipid vacuoles in paren- chymal cells		Necrosis in paren- chymal cells		Necrosis	
	Control	Methyl palm	Control	Methyl palm	Control	Methyl palm	Control	Methyl palm
7	(+)	(+)	-	-	-	-	+	+
14	+	+	-	+++	-	+++	++	+++
18	+	+	++	++	+	+	+++	+++

5. DISCUSSION

5.1. Methodological considerations

Because of its favorable physical characteristics, $^{99}\text{Tc}^{\text{m}}$ is a suitable radionuclide for clinical diagnostic purposes. It has a short half-life (6 hours), low radiation dose to the patient, favorable photon energy of 140 keV for scintillation camera imaging and is readily available from ^{99}Mo -generators.

The longer half-life, higher radiation dose and higher photon energy of ^{198}Au , make this isotope less suitable for experimental as well as clinical studies.

A method for preparation of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid for scintigraphy of the liver was first described by Harper et al (162). A simplified method of preparing $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid by use of a reduction of pertechnetate with acid, and by use of sodium thio-sulphate in the presence of a stabilizing agent has since been described (84, 85, 156, 164). To keep the preparation in colloidal form, gelatine, dextran or carboxymethylcellulose (CMC) have been used as stabilizers. The $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid used in the present study, prepared according to Persson and Naversten (156) has been used extensively without side effects in clinical practice for scintigraphy of the liver and spleen. Since different methods of preparation of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid may yield colloid particles of different size, with different extraction efficiencies (85), it is of fundamental importance to characterize each colloid preparation. The importance of a highly standardized colloid for estimation of RE function has been stressed by Pirttiaho and Pitkänen (165). In the present study the labelling efficiency was tested in each of the experiments with gel chromatography column scanning. This method may also give indications of the particle size and activity distribution (86, 165). Estimation of particle size of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid was performed with light microscopy and electron microscopy by Persson and Naversten (156). In a light microscope, the mean particle size was estimated to be 600 ± 200 nm ($\pm$ SD), whereas using the electron microscope, mean size was 800 ± 200 nm. Because of the small size of the particles, light microscopical studies were inappropriate. Electron microscopy photographs of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid were reported by Persson et al (157), showing the particles as large and small irregular shaped flakes. However, subsequent examination of the colloid using scanning electron microscopy (86) demonstrated well-defined, spherical particles. The differences may be explained by different fixation techniques. Because of these variations in results, it is important to combine morphological observations with other methods for determining particle size. The polycarbonate film filters (Nuclepore[®]) have been shown to be superior to the cellulose membrane filters (Millipore[®]) in determining the size of radiolabeled colloids (167). The mean particle size of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid, estimated by Nuclepore[®] filtration, was 300 nm

and, by scanning EM, 300-600 nm (86). These values were in good agreement with the values obtained from measurements by a Coulter Nano-Sizer®. As these measurements indicated increased particle aggregation with time, only freshly prepared colloidal suspensions were used in each experiment. The number of particles in the standard preparation used in the present study was estimated by empirical calculation to be about 10^{18} /ml (86).

In autoradiographic studies of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and ^{198}Au colloid, it was only possible to show internalization of the gold-colloid in Kupffer cells (168). Autoradiographic studies have shown sulphur particles adhered to but not ingested in Kupffer cells 24 hours after sulphur colloid administration (169). In the present study, however, morphological examination showed vacuolization of sinusoidal cells indicating an active endocytosis of the colloid particles (I, III).

When comparing results from studies using different colloid preparations it is of fundamental importance to be aware of the different characteristics governed by the colloidal particle size.

In a comparative study of ^{198}Au and $^{99}\text{Tc}^{\text{m}}$ -sulphur colloids, Mundschenk et al observed that the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid was accumulated in the spleen to a greater extent than the ^{198}Au colloid (96). Using preparations of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid with different particle size, smaller particles accumulated comparatively more in the bone marrow than particles of greater size (85). These observations were confirmed in the present study (III).

The greater uptake in the lungs of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid may be explained by a trapping of larger particles, since the injected colloid first passes the lung capillaries. The organ distribution of the $^{99}\text{Tc}^{\text{m}}$ - S_2S_7 colloid found in this study (III) was in good agreement with the results of a similar study by Frier et al (170).

As the blood flow to the organs influences the uptake of colloids, especially when small doses of colloids are administered, this factor must be taken into account when interpreting differences in $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid uptake rates (82, 171, 172). Biozzi et al (72) demonstrated that small doses of colloids were extracted from the blood exclusively by the liver. By increasing the dose, an uptake in the extrahepatic RES could be demonstrated. In the present study, an accumulated extrahepatic uptake of 3-15 % was demonstrated (I, II). Such variations may be due to variations in particle size and number of injected particles (85). This suggests that the employed dose was sufficient to make the uptake rate not exclusively dependent on organ blood flow.

Using Wistar rats and the same tumors as in the present study, Sundqvist et al (173) investigated, with a microsphere technique, the

cardiac output and organ blood flow in tumor-bearing animals. These studies revealed no changes in cardiac output or blood flow to the liver and spleen in animals with liver or subcutaneous tumors. In animals with liver tumors, the blood flow in the normal liver parenchyma surrounding the tumor was unaffected. It thus seems improbable that changes in liver blood flow of tumor-bearing animals account for the differences in colloid uptake rate observed in the present study (II).

The concept of the "critical dose" has been challenged by many investigators (22, 174). Jaques (174) going back to Biozzi's data (72), has demonstrated the invalidity of Equations 1 and 2. Standard procedures of curve fitting revealed that extraction curves were not exponential, and that the product of dose by extraction rate constant was not dose-dependent. Colloid uptake by the RES in rats has been shown to be fast, with plasma half-time values of 1-3 min (175). Because of this rapid uptake, sequential measurements at short time intervals are required for rate calculations. Since the uptake rate differs at various intervals from the start of injection, a modification was performed, allowing more measuring points on the initial phase of the uptake curve (II). In the formula for k_{phag} determination (Eq 1), the activity content is determined at two specific times requiring a small measuring point uncertainty at each time. By injecting sufficiently high activity, however, low enough to avoid dead time losses in the scintillation camera, the statistical uncertainty was kept at a low level in each time interval (I). When the measurements were being made, about 9.3 MBq was injected giving about 14 000 counts per 15 s or a standard deviation of 0.8 % (I). The phagocytic index, k_{phag} , which was derived from the uptake curve of the colloid in the liver was calculated as the logarithm of the quotient of the uptake values at two different times, i.e., 60 s and 120 s after injection. During this period, the count rate of the liver almost doubled. To demonstrate the dependence of k_{phag} on the k_1 and k_2 values, the following calculations were made:

$$k_{phag} = \log L(120) - \log L(60) \quad (12)$$

or:

$$k_{phag} = \log \frac{L(120)}{L(60)} \quad (13)$$

The activity uptake of the liver at 60 s, $L(60)$ and at 120 s, $L(120)$ could be calculated from Equation 9.

This results in:

$$k_{phag} = \log \frac{1 - e^{-(k_1 + k_2) \cdot 120}}{1 - e^{-(k_1 + k_2) \cdot 60}} \quad (14)$$

A slow uptake rate of the liver, i.e., low k_1 , combined with a fast uptake rate of the extrahepatic RES, i.e., high k_2 , thus would give the same value of k_{phag} as the opposite situation (high k_1 and low k_2). For very low values of (k_1+k_2) , k_{phag} approaches the limit $\log 2$ of 0.3010 (Fig 13). For very small values of (k_1+k_2) , Equation 14 could be written:

$$\lim_{(k_1+k_2) \rightarrow 0} k_{phag} = \lim_{(k_1+k_2) \rightarrow 0} \log \left[\frac{1 - \left(1 - \frac{120(k_1+k_2)}{1!} + \frac{[120(k_1+k_2)]^2}{2!} - \dots\right)}{1 - \left(1 - \frac{60(k_1+k_2)}{1!} + \frac{[60(k_1+k_2)]^2}{2!} - \dots\right)} \right] = \log 2 \quad (15)$$

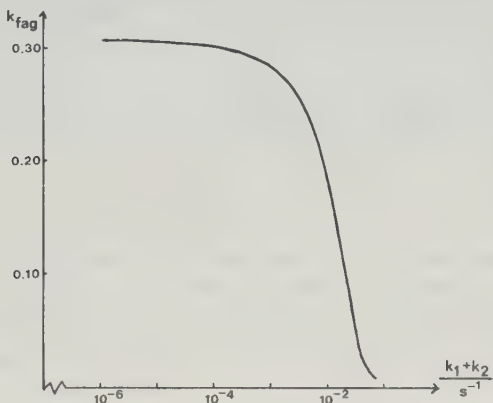


Fig 13. Relation between k_{phag} and (k_1+k_2) according to Equation 14.

In Figure 14, theoretical uptake curves have been plotted for different values of the rate constants k_1 and k_2 . The corresponding values of k_{phag} according to Equation 14 are also given.

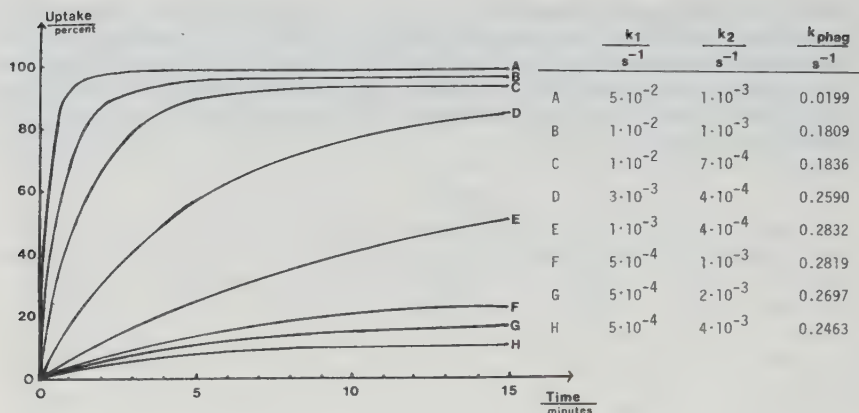


Fig 14. Theoretical uptake curves and the corresponding values of k_{phag} , k_1 and k_2 exemplifying the uncertainty of using the k_{phag} value as a measure of uptake rate.

It is thus obvious that selection of a time interval on the uptake curve by measuring the uptake at only a few different times will not provide a proper estimate of the uptake function in the organ. The same applies to the analysis of blood clearance curves. The present study emphasizes the inferiority of using Equations 1 and 2 for determination of RE function.

By using the compartment model, calculation of uptake was made using the whole uptake curve, giving enough measuring points for appropriate calculations. The uncertainty of using only a few measuring points in calculation of the phagocytic index was demonstrated by the wider distribution of the k_{phag} as well as the k_{phagCM} values compared with the k_1 values. Similar compartment models have been used for calculation of Kupffer cell function (81, 87, 94, 95, 176, 177, 178).

The plasma colloid clearance technique does not permit separate studies of the uptake by different RE organs. The technique applied in the present study, however, permits separate studies of hepatic and extrahepatic (mainly spleen) RE function. Suppression of colloid uptake in the liver is often accompanied by an increased colloid uptake in the extrahepatic RES (87). The depression of colloid uptake of the liver, induced by gelatine, was accompanied by an increased extrahepatic uptake rate in the present study (I). The depression of RE activity of the liver by methyl palmitate (V) was, however, not

accompanied by any significant changes in colloid uptake rate of the extrahepatic RES. The effects of gelatine and methyl palmitate were in accordance with previous reports (100, 106).

Several other colloids have been used for determination of RE function (Table II). Microaggregates of albumin have the advantage of allowing simultaneous studies of the colloid uptake as well as colloid breakdown or catabolism in the RE cells (81, 176, 177, 179). The use of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid does not allow for calculations of catabolism and it has been generally regarded as an inert colloid. Recent studies, however, suggest that sulphur particles may be broken down in vivo (170).

Studies comparing the uptake of sulphur colloid and aggregated albumin by the RES have revealed similar characteristics of the two colloids (180, 181). In studies comparing the efficiency as RE test substances of labeled sheep red blood cells (SRBC) with sulphur colloid, dextran sulphate impaired the uptake of SRBC but not of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid (103). Since the IgM-coated red blood cells are extracted by the immunospecific C3b receptor on Kupffer cells, while sulphur colloid probably is adhered to Kupffer cells by non-immunospecific opsonin-mediated mechanisms, this difference may explain the findings (42).

In order to investigate if a lysosomal enzyme, β -hexosaminidase, could serve as a marker for phagocytosis, plasma levels of this enzyme were determined after administration of different agents. These agents were either known to affect RE function (methyl palmitate, zymosan, alcohol, Haemaccel[®]), or were used as test substances of RE function ($^{99}\text{Tc}^{\text{m}}$ -sulphur colloid, ^{198}Au colloid) (III). Cultured human macrophages derived from peripheral blood monocytes have been shown to release β -hexosaminidase in response to serum-opsonized zymosan (123). After the injection of zymosan, serum activity of certain lysosomal enzymes such as β -glucoronidase and acid phosphatase was increased (114). These findings were confirmed in the present study concerning β -hexosaminidase. Turnover of rat β -hexosaminidase has been shown to be very short (0.4 days) when purified preparations (liver, epididymis) were intravenously injected (68). This may explain the normal values of β -hexosaminidase 24 h after zymosan injection (III).

Endocytosis of zymosan has been shown to be very rapid and morphological studies have shown that this occurs primarily in lung macrophages but zymosan is later found in Kupffer cells (114). The present results of the histological examinations of lung and liver also suggest that the uptake in the lung precedes the liver uptake (III). This could be an explanation for the two-step rise in plasma β -hexosaminidase activity (Fig 9) seen after intravenous zymosan injection (III).

A variety of particulate and soluble stimuli from chronic inflammation trigger synthesis and release of lysosomal enzymes in macrophages

(117, 182, 183, 184). In the present study, none of the injected colloids caused significant alterations in plasma levels of β -hexosaminidase. This may be explained by the fact that these particles during the time of study, merely adhere to the macrophages or by the fact that particles are taken up primarily by Kupffer cells which cells are also known to clear lysosomal enzymes from the blood (68, 126). The differences in particle size of the injected colloids did not influence lysosomal plasma levels (Table XII) (III).

Alcohol may depress Kupffer cell phagocytic activity as measured by the clearance of microaggregated albumin (185). Although increased levels of β -hexosaminidase were found in patients with acute alcohol intoxication (186), alcohol may not be the only responsible factor. In the present study, no change in β -hexosaminidase activity was found after a single intravenous injection of alcohol (III), nor did alcohol cause a further rise in enzyme levels after zymosan injection (IV).

No signs of phagocytosis of methyl palmitate have been observed after intravenous administration (101). These results are in accordance with the present histological examination which revealed no signs of destruction of RE cellular elements after methyl palmitate infusion (V). Administration of methyl palmitate interferes with Kupffer cell phagocytic function as revealed in the present study (V) and by others (100, 101, 102).

A reduction in the RE phagocytic capacity after trauma has been correlated with increased plasma levels of cathepsin and acid phosphatase in rats (187). This has been explained by the ineffective clearance from the blood due to a decrease in Kupffer cell function. In the present study, the basal enzyme levels of methyl palmitate treated rats did not differ from those of control animals; neither did the enzyme response to zymosan differ in methyl palmitate treated rats compared with controls (IV). Administration of methyl palmitate did not influence the levels of β -hexosaminidase or LD as measured at the day of tumor inoculation (V). It thus seemed that basal levels of β -hexosaminidase are not indicative of the degree of activation of the macrophage system. However, the amount of enzyme released by a standard dose of zymosan may be correlated to the number of elicited macrophages as enzyme levels were higher in zymosan pretreated rats (IV).

These results were in accordance with the observation in mice that the release of β -hexosaminidase after administration of immunoadjuvants was related to the degree of macrophage activation (188). Tanner et al also found that both intracellular levels and release of β -hexosaminidase were increased from liver macrophages isolated from *Corynebacterium parvum* treated rats (189). The observations (IV) suggest that plasma levels of β -hexosaminidase after zymosan injection are more dependent on the degree of macrophage activation than on lysosomal enzyme clearance.

5.2. Reticuloendothelial function and tumor growth

Several studies on the RE function in association with experimental tumor growth have not discriminated between hepatic and extrahepatic RES (62, 130, 190, 191, 192). Studies of the uptake of ¹⁹⁸Au colloid in the liver and spleen of rats with lymphoma revealed an increased liver uptake whereas the total colloid uptake of the spleen was unaffected (128, 193). Enlargement of the spleen in animals with experimental tumors was, however, recognized early and attributed to an increased number of RE cells (194). In the present study (II), the applied technique permitted separate studies of the function of the hepatic and extrahepatic RES. Small tumors of similar size, located in the liver or in the dorsal subcutaneous tissue, independent of localization, were associated with increased colloid uptake rate of the liver as well as of the spleen. A correlation between total organ uptake rate and the accumulated activity of the organ was not invariably seen. The colloid uptake rate may be more influenced by organ blood flow and circulating opsonins, while the accumulated activity more reflected the total number of macrophages (10). A significant increase in spleen weight was noted in all groups of tumor animals and coincided with an increased accumulated activity in the spleen.

However, in the group with large retroperitoneal tumors, despite the increased weight and total accumulated activity of the spleen, the uptake rate of the extrahepatic RES was not different from controls but significantly decreased compared to animals with smaller tumors. In this group, the uptake rate of the liver was significantly decreased compared to controls. These observations probably reflect a decrease of humoral recognition factors (opsonins) in animals with large tumors (34, 192). A decrease in opsonin levels has also been reported in patients with cancer (35, 64, 145).

The accumulated activity of the liver was lower in tumor animals, regardless of tumor localization. This was not related to changes in the total weight of the livers. The uptake rate of the liver was correlated neither to liver weight nor to the total accumulated activity of the organ. The variations of uptake rate may thus again be explained by variations in humoral factors.

The effects on the growth of an experimental liver tumor by hepatic artery ligation and hyperthermia have been reported (195, 196). Using the same tumor model, the influence on tumor growth of RE suppression induced by methyl palmitate was studied (V). By injecting a tumor cell suspension into the periphery of the central liver lobe just under the capsule it was possible to produce a single ellipsoid liver tumor. The method of approximating tumor volume has been shown to be reliable and well correlated with the actual tumor volume (195). Earlier experience with the same liver tumor showed that more than 3 laparotomies in the 3 week post-inoculation period were associated with a high mortality of rats (195). Inoculation of liver tumors was performed at a time of

significant RE suppression, as revealed by the reduced hepatic uptake rate of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid. Other factors may influence RE phagocytic activity, such as numbers and duration of anesthetic procedures, laparotomies and blood samplings (107). These factors were carefully equalized in both methyl palmitate treated and control animals. Striking differences between the two groups were noted. Because of the small number of surviving rats in the methyl palmitate treated group at day 18, no conclusions regarding statistically significant differences could be made at that time.

Since intracellular levels of lysosomal enzymes have been shown to be increased in the liver of tumor-bearing mouse (197) and since activated macrophages may secrete these enzymes (69, 183), plasma levels of the lysosomal enzyme, β -hexosaminidase, were determined during the course of the experiment (V). It was not possible in the present study to show any difference at the 0.05 level of significance in plasma levels of β -hexosaminidase in response to tumor growth, neither was any difference apparent after methyl palmitate RE suppression. A difference may, however, exist between different lysosomal enzymes since glucan activated hepatic macrophages showed increased levels of, in particular lysozyme, while β -hexosaminidase was unaffected (198). Whether other lysosomal enzymes may be affected has to be tested in further studies.

LD release is usually considered to be an indication of cell damage and death. The release of β -hexosaminidase of elicited macrophages was not combined with LD release although a correlation was found between LD accumulation and cell loss of elicited macrophages (183). In the present study, LD release was not apparent in spite of histological evidence of necrosis of tumor and liver parenchyma.

Compared to the abundant literature concerning the effects of macrophage activation on tumor growth (66, 141, 142, 143, 148, 150, 199, 200), relatively little information is available regarding the opposite situation, i.e., the role of a depressed RES in tumor growth (63, 66).

Studies by North et al (146) have shown an increased susceptibility to infections by *L. monocytogenes* of mice, during the initial period after tumor inoculation. This early suppression of macrophage function may be important for subsequent tumor development (155). An accelerated growth of a subcutaneous adenocarcinoma in mice after methyl palmitate administration was reported by DiLuzio (66). In a study on the development of experimental lung metastases in Wistar rats, trauma enhanced the development of lung metastases. The combination of trauma and dextran 40 (factors known to depress RE function) was even more effective in this respect (201). The observed enhancement of tumor growth in the present study is well in accordance with these earlier findings.

It is a well experienced fact that patients with non-resectable carcinoma often demonstrate rapid deterioration and accelerated tumor growth after surgery. The initial RE suppression seen after surgical procedures may be of some importance in this context (10, 107, 154, 202).

5.3. Clinical considerations and future aspects

The temporary RE depression following surgery has been correlated with opsonin depletion (107, 203). In a clinical study, Aronsen et al (204) found temporarily decreased levels of cold insoluble globulin after cholecystectomy. Obviously this "physiologic" RE depression is seldom of clinical significance. However, it may be a critical factor in otherwise deteriorated patients, such as patients with advanced cancer or serious infection. In addition, compounds such as plasma substitutes and heparin, used widely in clinical practice, have been shown to negatively affect the RE function (106, 175). Ultrastructural studies have shown that artificial lipid emulsions, e.g., Intralipid®, attach strongly to Kupffer cells whereas chylomicrons do not (16). Removal of lipid particles by the RES has been reported in humans receiving Intralipid® (205). Intralipid® has also been shown to impair bacterial clearance and enhance bacterial virulence in mice (206).

Few studies have been carried out in humans, correlating the functional status of the RES to the clinical outcome of the patient. Scovill et al (1978) reported on the results of replacement therapy with cold insoluble globulin in cryoprecipitate after injury with documented hypopopsonemia (207). In all 6 patients, the severe opsonin depletion was reversed and all patients had rapid improvement in febrile state, normalization of leukocyte levels and improvement in pulmonary function.

Although these results have been difficult to duplicate, they point to the importance of the RE function in critically ill patients. Lack of simple and reliable methods to determine the RE function has certainly limited our knowledge of the importance of agents affecting RE function. It seems logical, however, that in situations with an already depressed macrophage function, substances that further hamper RE function should not be administered.

The clinical importance of a depressed RES in association with tumor surgery needs further clarification, as our results show that even a short period of RE depression may influence tumor growth. Although the liver is the main stationary RE organ, it is also the most common site of distant metastatic involvement from intestinal tumors (208). It is well known that surgical manipulation of colonic carcinomas give rise to increased levels of tumor cells in the portal blood (209). A concomitant depression of the macrophage function in the liver may be important for the further fate of these cells. It seems reasonable

therefore to avoid further RES depression during and immediately after cancer surgery.

The critical role of hepatic RE function for liver regeneration has been demonstrated by Holper et al (210). Their results demonstrated that impaired phagocytosis and possibly other functional alterations of Kupffer cells played a significant role in the pathophysiology of acute hepatic failure. Derangement in RE function was most indicative of the final outcome of hepatic ischemia, in contrast to measurements of bilirubin, bromsulphalein (BSP), liver transphases, lactic dehydrogenase and alkaline phosphatase. Therefore it seems that measurements of RE function could be of prognostic value in assessing liver function in patients with severe liver disease or after severe liver trauma. Whether stimulation of RE function in this situation could influence the outcome of these patients ought to be evaluated as well as the importance of other factors which deteriorate RE function.

Although the focus of interest in this work has been the RES as a host defense system, the macrophage system interacts in many physiologic and pathophysiologic functions of fundamental importance (Table I).

Disturbances of RE function may thus take many forms: altered carbohydrate-, lipid- and protein metabolism; decreased resistance to infection and shock; disturbances in immunologic processes and disturbances in hemostasis. In view of these various functions the clinical indications for assessment of RE function seem almost infinite. In an equal number of situations the possibility of stimulating RE function would be of significant interest. In spite of this no simple and reliable method exists for measuring RE function in man. The need for technical equipment such as a scintillation camera and computer systems has been an obstacle to routine clinical use. However, mobile scintillation cameras are available. Another drawback is the fact that the phagocytized test particles may negatively influence RE function at least temporarily. The possibilities of the present technique will be evaluated in clinical studies in combination with other methods for measurements of RE function such as opsonin assessment and analyses of lysosomal enzymes.

6. SUMMARY AND CONCLUSIONS

With the applied technique, based on the same technical facilities as routine liver-spleen scintigraphy, it has been possible to demonstrate alterations of colloidal uptake rate of the hepatic and extrahepatic RES of experimental animals. These findings have been correlated to the RE function, and earlier results concerning RE function after administration of various agents known to suppress RE function have been reproduced with this method. The colloid preparations used have been thoroughly characterized and the measuring technique has the advantage of non-invasive registrations. Alterations in RE function during different stages of tumor growth have also been detected.

The possibilities of using the plasma levels of a lysosomal enzyme, β -hexosaminidase, as markers of RE function were elucidated. Basal enzyme levels were not correlated to the degree of RE function. After zymosan injection the increase of enzyme levels may be an indication of the degree of activation of the macrophage system.

The administration of a reticuloendothelial suppressing agent (methyl palmitate) was found to enhance the growth of an experimental liver tumor and significantly influence mortality after tumor inoculation. This study points to the importance of the macrophage system in host resistance against tumors.

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A Scintillation Camera Technique for Measurements of the Reticuloendothelial Function – Comparison of Different Methods for Measuring RES Function

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Abstract. A highly standardized ^{99m}Tc-sulphur colloid was used to evaluate reticuloendothelial system (RES)-function in the normal rat and after RE blockade by gelatin (Haemacell). Activity distribution in the animals was measured with a scintillation camera technique. Total uptake of activity in the liver was estimated. From the time-activity curves over the liver, the phagocytic index (k_{phag}) was evaluated. Estimation of the uptake rate of the labelled colloid into the liver and into other parts of the RES was also performed using a two-compartment model. Different methods of evaluation of RE function were compared. It was shown that for a proper estimation of the RE function, the whole uptake curve must be considered. Gelatin (Haemacell) significantly reduced the total colloid uptake by the liver. The colloid uptake rate into the liver was also significantly reduced. Liver specimens after colloid injection were examined by light and electron microscopy showing vacuolation of hepatocytes and sinusoidal cells probably due to pinocytosis. The technique described enables functional studies of the RES. It has the advantage of non-invasive registrations and is based on the same technical facilities as used for routine liver scintigraphy.

by the uptake of injected radio-labelled colloid particles by the liver. The method was evaluated in normal rats as well as in rats after gelatin (Haemacell) infusion, which is known to depress the RE function (Schlitt et al. 1975).

Material and Methods

Test Substance

The test substance, ^{99m}Tc-sulphur colloid (99^mTc-S₂), was prepared from 48 μmol sodium thiosulphate (Na₂S₂O₄ · 5H₂O) and 9 μmol potassium persulfate (K₂ReO₄) according to Persson and Naversten (1970). The particle size of the colloid was 600 ± 200 nm. The preparation was tested in each of the experiments for the quality of the colloid, i.e. the labelling efficiency, with gel chromatography column-scanning technique (Persson et al. 1978; Strand 1979). The injected activity was approximately 9.3 MBq (250 μCi). A typical activity distribution on a Sepharose 4B column for the ^{99m}Tc-S₂ colloid is depicted in Fig. 1.

Animal Preparation

Female Wistar rats (150–200 g), fed on unrestricted standard food pellets and water, were used in all experiments. Under general anaesthesia with Nembutal 6 mg/kg, the jugular vein was cannulated and a fine catheter was introduced and fixed with its tip in the superior caval vein. Ten control rats were not pretreated and five rats were subjected to pretreatment with IV gelatin (Haemacell, MW = 35,000) infusion (1.3 ml/10 min) prior to registration. With the animal fixed under the scintillation camera, 0.5 ml test colloid was injected during 5–10 s and registration started immediately at the time of injection.

After completion of the scintillation camera registration the animals were killed. Liver specimens were taken from eight rats not pretreated with gelatin and killed 1, 3, 5, 10, 30 and 60 min and 24 h and 48 h after administration of the colloid. For light microscopy, slices were fixed in 4% formaldehyde, embedded in paraffin and stained with Hematoxylin-erythrosine, van Gieson, the Turnbull-blue reaction for iron and with Fouchet's reagent for bilirubin pigment.

For electron microscopy, slices were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and 0.1 M sucrose, pH 7.2, at +4°C for 4 h with post-fixation in 2% OsO₄ in 0.1 M cacodylate buffer, pH 7.4, at +4°C for 1 h. The slices were embedded in Epon and 1-μm sections were stained with meth-

Introduction

Reticuloendothelial (RE) function plays a major role in host defence, not only against infection and foreign matter but also against malignant disease (Old et al. 1961). The reticuloendothelial system (RES) constitutes a system of fixed and mobile macrophages, widely scattered throughout the body, in close contact with the circulating blood, and with the ability of phagocytosis as a common denominator. The most important stationary RE organ is the liver, constituting about 80% of the total fixed RE capacity (Saba 1970).

The ability to clear colloid particles from the bloodstream has been used for the determination of RE function for several years. A mathematical expression for the clearance of inert particles by the RES was defined by Biozzi et al. (1953). Further knowledge about the nature of the colloid disappearance curves was contributed by Dobson and Jones (1952). The use of radio-isotope labelled colloids has made possible simultaneous studies of the clearance from the blood as well as the uptake of the colloid by different RE organs (Stern et al. 1966).

The aim of this study was to develop a simple and reliable method of determining the RE function in rats as expressed

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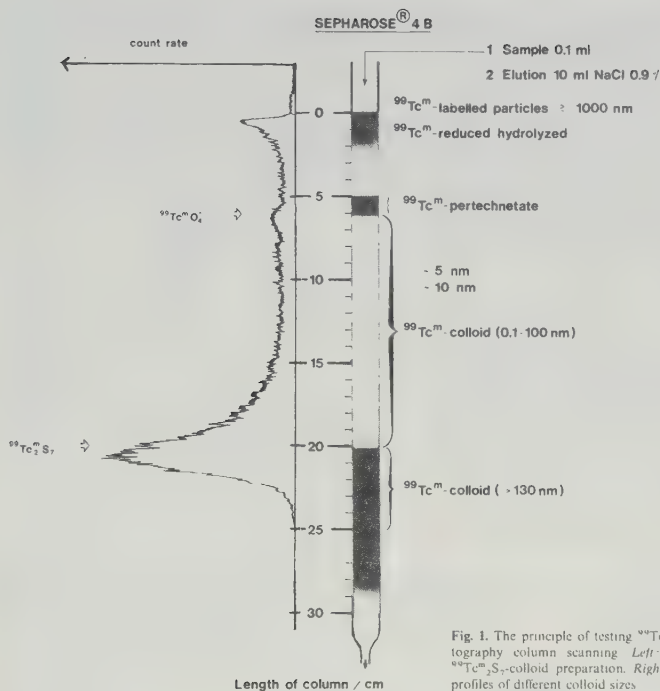


Fig. 1. The principle of testing $^{99}\text{Tc}^m$ -sulphur colloid by gel chromatography column scanning. *Left:* the count rate curve from a $^{99}\text{Tc}^m\text{S}_2$ -colloid preparation. *Right:* the localization of the activity profiles of different colloid sizes.

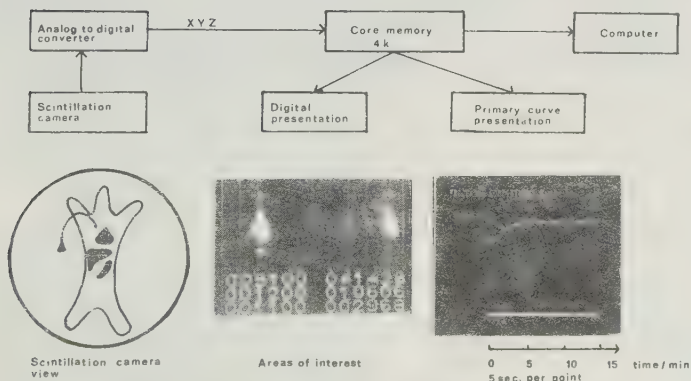


Fig. 2. Scintillation camera and data acquisition scheme. Scintigraphic picture of liver, spleen and heart of a normal rat. To the right the corresponding time-activity curve of the liver and the curve fitted according to Eq. (4).

ylene blue-thionin. Ultrathin sections were contrasted with uranyl acetate-lead citrate and examined in a Zeiss EM10 electron microscope.

Scintillation Camera Technique

The kinetics of the activity distribution in the animals were measured with a scintillation camera (Pho/Gamma III HP, Searle Radiographic Inc.), equipped with a parallel 16000 hole-collimator (Fig. 2). Sequential images were taken every 15 s from the start of injection and continued for 15 min. Data were stored on magnetic tape. A region of interest in the liver was selected in the image and time activity curves were generated (Fig. 2).

Computer Analysis

The time activity curves were punched on a paper tape and transferred to a computer (Digital Equipment PDP 11/45) where uptake curves were evaluated. In order to calculate the percentage uptake of the activity in the liver $P(t)$, a calibration of the sensitivity of the camera was necessary. Using a water tank and having the ^{99m}Tc activity in rat liver phantom, the calibration factor, α , i.e. the count rate per activity unit, could be calculated and was shown to be

$$\alpha = 0.10 \text{ s}^{-1} \text{ kBq}^{-1} (3.7 \text{ s}^{-1} \mu\text{Ci}^{-1})$$

If $A(0)$ is the activity injected and $N(t)$ the net number of counts in the liver region in the image, the percent uptake in the liver $P(t)$ can be expressed

$$P(t) = N(t) \cdot \{\alpha \cdot \Delta t \cdot A(0)\}^{-1} \cdot \exp\{\lambda \cdot t\} \cdot 100 \quad (1)$$

where t is the time after injection, Δt is the frame time and λ the physical decay constant for ^{99m}Tc .

$$\lambda = \ln 2 \cdot 6.02 \text{ h}^{-1} \cdot 0.12 \text{ h}^{-1}$$

To estimate the flow rate of the labelled colloid into the liver an open two-compartment model was used (Fig. 3). With the symbols in the figure, the rate of change of the colloid in the blood, B , and in the liver, L , with time can be expressed:

$$\frac{dB}{dt} = -B(k_1 + k_2) \quad (2)$$

$$\frac{dL}{dt} = B \cdot k_1 \quad (3)$$

The amount of colloid transported to the liver can thus be expressed by the equation:

$$L(t) = 100 \cdot \frac{k_1}{k_1 + k_2} \cdot \{1 - e^{-(k_1 + k_2)t}\} \quad (4)$$

The rate constants k_1 and k_2 were obtained by fitting this equation to the experimental liver uptake curve for each animal (Fig. 2).

Models for Evaluation of RE Function

The liver uptake curve shows the pattern of an initial rapid component, an intermediate component and a slower 'tailing' component (Fig. 2). The uptake by the RES of colloid particles is mostly described either by the phagocytic index, k_{phag} or by the half-time of clearance from the blood $T_{1/2}$ (Saba 1970). The first very rapid component of the curve represents

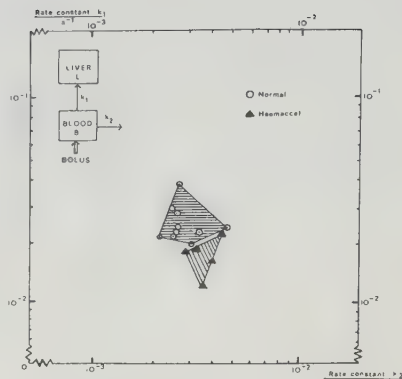


Fig. 3. Scatter diagram of the rate constants k_1 and k_2 obtained from the two-compartment analysis. Normal rats and rats after pretreatment with Haemacel infusion

Table 1. Total liver uptake of ^{99m}Tc sulphur colloid in normal and gelatin pretreated rats, in percentage of the injected colloid dose

	n	% Activity		
		Range	Mean	
Normal	10	84.7-97.2	91.2	$P < 0.025$
Gelatin	5	78.1-90.7	83.7	

Statistical analysis for 95% confidence by Student's t -test.

both mixing of the test substance with blood and the initial phagocytic uptake during the first 40-60 s after injection. This component is thus less suitable for calculating the phagocytic function. The second, intermediate part of the curve represents further phagocytosis of the colloid in the organ in question. Blood mixture of the colloid can now be assumed complete and the second part of the curve is thus more appropriate for calculation of phagocytic function. The slow, 'tailing' component may be related in part to the heterogeneity of colloid particle size, smaller particles being phagocytosed at a slower rate than larger particles (Scott et al. 1967). The phagocytic index k_{phag} is calculated in the second part of the curve using the formula:

$$k_{\text{phag}} = \log \frac{A(120)}{A(60)} \quad (5)$$

where $A(120)$ is the uptake of activity (i.e. proportional to the number of particles phagocytosed) in the liver at 120 s after injection. $A(60)$ is the uptake at 60 s. During this interval (60-120 s) the activity in the liver almost doubles.

The k_{phag} values were also determined from the compartment analysis (k_{phagCM}) by putting the obtained k_1 and k_2 values into Eq. 4 and thus the activity in the liver could be determined at any time after the injection. The accumulated activity in the liver and spleen, during 15 min of registration

was also calculated. Correction for the physical decay of ^{99m}Tc was made.

Statistics

Statistical analysis of total liver uptake of colloid was performed for 95% confidence by Student's *t*-test (Table 1). Analysis of k_1 - k_2 values from the compartment analysis (Fig. 3) was done by computing a single best-fit linear relationship between k_1 and k_2 by a technique described by Barlett (1949).

Results

The activity distribution and the time-activity curve for a normal rat are depicted in Fig. 2. The accumulated activity in the liver for normal and Haemaccel treated rats is given in Table 1. This shows a significant reduction ($P < 0.025$) in the liver uptake for Haemaccel pretreated rats in comparison with normal rats.

The k_{phag} , k_{phagCM} and k_1 values for normal rats are given in Table 2. Comparison of k_{phag} with k_{phagCM} reveals no significant difference in the range of values and the standard deviation was the same. A comparison between k_{phag} and k_1 shows that k_1 values are more narrowly distributed with less deviation from the mean value.

k_1 and k_2 values for normal and Haemaccel treated rats are given in Table 3 and Fig. 3. Haemaccel significantly re-

Table 2. Comparison of different methods for evaluation of liver phagocytosis in normal rats

	$\bar{x}$	Range	SD	
k_{phag}	0.18	0.11-0.24	0.04	22%
k_{phagCM}	0.21	0.15-0.32	0.05	23%
$k_1 \cdot 10^{-1}$	0.23	(0.19-0.29)	0.03	13%

Table 3. k_1 and k_2 values for normal and Haemaccel treated rats

	Normal $n = 10$	Haemaccel $n = 5$
$k_1 \cdot 10^{-1}$	(0.23 $\pm$ 0.03)	(0.18 $\pm$ 0.04)
$k_2 \cdot 10^{-2}$	(0.30 $\pm$ 0.05)	(0.35 $\pm$ 0.05)
	$P < 0.01$	

duced the rate of colloid uptake in the liver as estimated by the compartment analysis ($P < 0.01$).

Light microscopy showed vacuoles in the hepatocytes, especially in the central parts of the lobuli, from 1 min to 24 h, most pronounced from 3 to 30 min. The vacuoles were often found in rows in the periphery of the cells close to the sinusoids (Fig. 4a). In electron microscopy the vacuoles were often found to be surrounded by a membrane and to contain a floccy substance (Fig. 4b, c). No pathological changes could

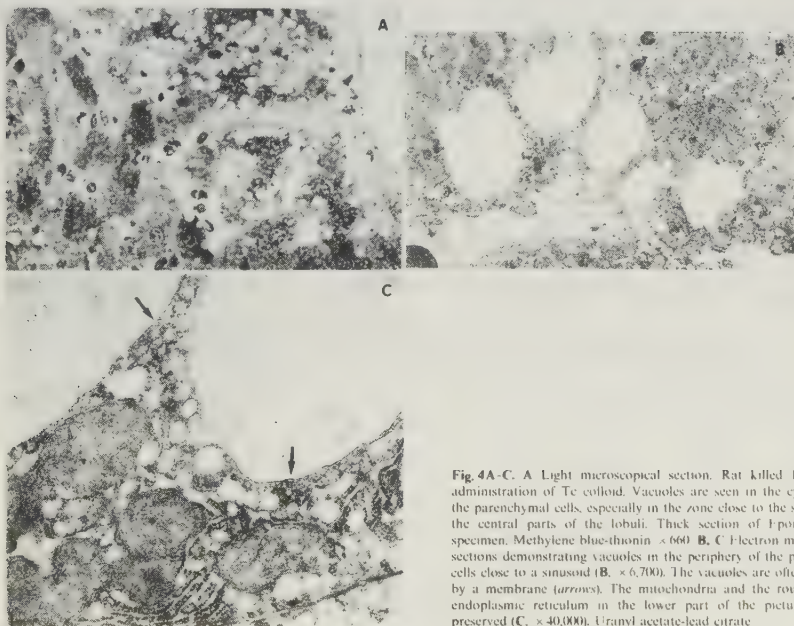


Fig. 4A-C. A Light microscopical section. Rat killed 10 min after administration of Tc colloid. Vacuoles are seen in the cytoplasm of the parenchymal cells, especially in the zone close to the sinusoids, in the central parts of the lobuli. Thick section of Epon-embedded specimen. Methylene blue-thionin $\times 660$. B, C Electron microscopical sections demonstrating vacuoles in the periphery of the parenchymal cells close to a sinusoid (B, $\times 6,700$). The vacuoles are often delimited by a membrane (arrows). The mitochondria and the rough-surfaced endoplasmic reticulum in the lower part of the picture are well preserved (C, $\times 40,000$). Uranyl acetate-lead citrate

be ascertained in other parts of the cells. Vacuoles were also found in large amounts in the sinusoidal cells.

Discussion

^{99m}Tc sulphur colloid has been widely used for estimation of RE function (Harper et al. 1965; Atkins et al. 1970; Houston and Macleod 1980). The colloid uptake by the RES in rats has been shown to be fast, with plasma half-time values of 1–3 min (Berghem et al. 1978). After 15 min, more than 85% of the given activity was distributed to the liver. Pretreatment with Haemacel significantly reduced the total liver uptake of colloid. This is in accordance with other studies (Schildt et al. 1975). As shown in Fig. 2 the main uptake occurs during the first 5 min after injection. After 10 min there is only a very slight further uptake by the liver. Because of this rapid uptake, sequential measurements at short intervals are required for rate calculations. In the formula for k_{phag} determination, the activity content is to be determined at two specific times and the measuring point uncertainty at each time has to be as small as possible in order not to obtain k_{phag} values scattered over a wide range of values. By injecting high enough activity, it was however, low enough to avoid any 'dead time'

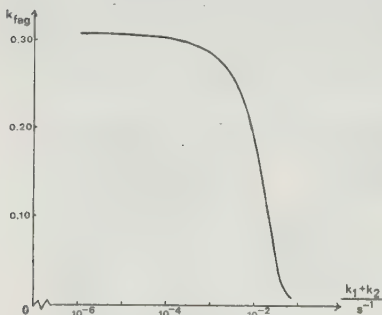
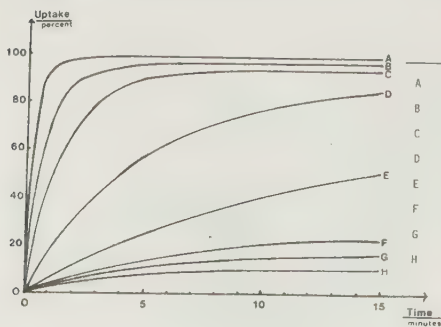


Fig. 5. Relation between k_{phag} and $k_1 + k_2$ according to Eq. (11) see text



losses in the scintillation camera, the statistical uncertainty was kept at a low level in each time interval. When the measurements were being made, about 9.3 MBq (250 μCi) was injected giving about 14,000 counts per 15 s or a standard deviation of 0.8%.

The phagocytic index, k_{phag} , which is derived from the uptake curve of the colloid in the liver, is calculated as the logarithm of the quotient of the uptake values at two different times, i.e. 60 s and 120 s after injection.

k_{phag} can also be evaluated from the equation:

$$k_{\text{phag}} = \log \frac{L(120)}{L(60)} \quad (6)$$

or:

$$k_{\text{phag}} = \log \frac{L(120)}{L(60)} \quad (7)$$

$$L(t) = 100 \cdot \frac{k_1}{k_1 + k_2} \cdot [1 - e^{-(k_1 + k_2) \cdot t}] \quad (8)$$

Thus:

$$L(120) = 100 \cdot \frac{k_1}{k_1 + k_2} \cdot [1 - e^{-(k_1 + k_2) \cdot 120}] \quad (9)$$

$$L(60) = 100 \cdot \frac{k_1}{k_1 + k_2} \cdot [1 - e^{-(k_1 + k_2) \cdot 60}] \quad (10)$$

This gives:

$$k_{\text{phag}} = \log \frac{1 - e^{-(k_1 + k_2) \cdot 120}}{1 - e^{-(k_1 + k_2) \cdot 60}} \quad (11)$$

It is obvious from this equation that k_{phag} is only dependent on the value of $(k_1 + k_2)$.

A slow uptake rate, i.e. low k_1 and high k_2 , will give the same value of k_{phag} as a very rapid uptake rate, i.e. high k_1 and low k_2 .

For very low values of $(k_1 + k_2)$, k_{phag} approaches the limit 0.3010 or $\log 2$ (Fig. 5).

For very small values of $(k_1 + k_2)$, Eq. (11) can be written:

$$\lim_{(k_1 + k_2) \rightarrow 0} k_{\text{phag}} = \lim_{(k_1 + k_2) \rightarrow 0} \log \left[\frac{1 - \left(1 - \frac{120(k_1 + k_2)}{1!} + \frac{[120(k_1 + k_2)]^2}{2!} - \dots \right)}{1 - \left(1 - \frac{60(k_1 + k_2)}{1!} + \frac{[60(k_1 + k_2)]^2}{2!} - \dots \right)} \right] = \log 2. \quad (12)$$

Fig. 6. Theoretical uptake curves and the corresponding values of k_{phag} , k_1 and k_2 exemplifying the uncertainty of using the k_{phag} value as a measure of uptake rate

In Fig. 6 the uptake curves for the colloid in the liver are plotted for different values of the rate constant k_1 and k_2 . Also given are the corresponding values of k_{phag} .

A very rapid uptake, i.e. a very high value of k_1 will give a low value of k_{phag} . A slow uptake, i.e. a low value of k_1 can give a high value of k_{phag} . From these facts, it is obvious that selection of a time interval on the uptake curve, by measuring the uptake only at a few different times will not give a proper estimation of the uptake function in the organ.

By using the compartment model described, calculation of uptake was done using the whole uptake curve giving enough measuring points for appropriate calculation. The uncertainty of using only a few measuring points in calculation of the phagocytic index was shown by a wider distribution of the k_{phag} as well as the k_{phagCM} values compared with the k_1 values.

The Haemacel treated rats showed a significant ($P < 0.025$) reduction of total colloid uptake by the liver in comparison with normal rats. The compartment analysis also revealed a significant ($P < 0.01$) reduction of the colloid uptake rate by the liver in the animals treated with Haemacel.

This is consistent with a report by Schildt et al. (1975). This RE blocking effect was shown as early as 10 min after infusion of gelatin. There was no change in the uptake by the spleen after treatment with Haemacel, nor was any significant change in k_2 values noticed. Thus the reduction of uptake by the liver was not combined with an increased uptake by other parts of the RES.

The vacuoles found on microscopical examination (Fig. 4a-c) probably arise by pinocytosis. They are similar to those described after administration of another colloid, 1-ethenyl-2-pyrrolidinone polymers (Hübner 1968).

The importance of a highly standardized colloid for estimation of RE function has been stressed (Pirttiäho and Pitkänen 1977). This has been considered in the present study by testing the quality of the colloid in each experiment.

Similar compartment models have been used for the evaluation of Kupffer cell function using the plasma colloid clearance technique (Boisvieux and Steimer 1979). The technique described enables separate studies of the uptake by the two main stationary RE organs, liver and spleen. The compartment model also enables specific evaluation of the liver uptake rate in relation to the uptake rate of the other parts of the RES.

The technique involving the test substance ^{99}Tc -sulphur colloid, and the scintillation camera imaging, is based on the same basic technical facilities as used for routine liver scintigraphy. The technique has the advantages of non-invasive registrations and will be tested in patient studies.

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Reticuloendothelial Function in Normal and Tumor-bearing Rats. Measurements with a Scintillation Camera Technique*

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Abstract—The function of the reticuloendothelial system (RES) was evaluated after the inoculation of an experimental tumor in rats. Four groups were studied according to tumor size and site. Reticuloendothelial function was evaluated by measuring the biokinetics of a standardized [$^{99}\text{Tc}^m$]-sulfur colloid. Estimation of the uptake rate of the labeled colloid into the liver and other parts of the RES was performed through the use of a two-compartment model. Animals with small liver or subcutaneous tumors showed an increased activity of both the hepatic and the extrahepatic RES. Animals with large retroperitoneal tumors showed a significant decrease in the RE function of the liver. In these animals the function of the extrahepatic RES was not changed compared to controls but was, however, significantly decreased compared to animals with smaller tumors. The findings may reflect a difference in the impact of tumor size on RE function extra- and intrahepatically.

INTRODUCTION

THE RETICULOENDOTHELIAL system (RES) has many important functions in both physiological and pathological conditions. The importance of the RES in defence against tumor growth and spread has been documented in previous studies [1-5].

The Kupffer cells of the liver constitute the major part of the fixed macrophages, comprising about 80% of the fixed RES capacity. The spleen contributes another 10% to this total fixed macrophage capacity [6].

Tumor transplantation in experimental animals causes a rapid increase in reticuloendothelial activity, as measured by the blood clearance of colloid particles [2, 3]. A rapid increase in the weight of the spleen during experimental tumor growth has also been reported [2, 3]. The influence of RES activity on the growth of tumors has been described earlier [7, 8]. Suppression or stimulation of reticuloendothelial function has been shown to interfere

with tumor growth [9, 10]. It is also evident that surgical trauma depresses reticuloendothelial function [6, 11]. The clinical importance of these facts is still poorly understood.

In previous studies correlating reticuloendothelial activity to tumor growth, total RES activity as measured by colloid blood clearance has mainly been used [12]. The use of radiolabeled colloids has made possible simultaneous studies of the clearance from the blood and the uptake of the colloid by different RE organs [13]. These techniques allow the performance of separate studies of the uptake by the two main stationary RE organs, the liver and the spleen.

In a previous study we investigated the efficiency of a scintillation camera technique for measurements of the reticuloendothelial function, using a [$^{99}\text{Tc}^m$]-sulfur colloid test as the test substance [14]. This study was undertaken to evaluate the reticuloendothelial phagocytic capacity in rats with different degrees of intra- and extrahepatic tumor load.

MATERIALS AND METHODS

Inbred Wistar rats of both sexes were used in the experiment. All rats were fed *ad libitum* on rat pellets and water.

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Twelve rats were used as controls and 21 rats were used as recipients of an *N*-methyl-*N*-nitrosoguanidine-induced adenocarcinoma. The tumor was transplanted under sterile conditions into either the liver, kidney or dorsal subcutaneous tissue. Tumor transplantation to the liver and the subcutaneous tissue was done with 1.0×10^6 cells and to the kidney with 0.5×10^6 cells. Vital cell counts were made after adding trypan blue. The tumor-bearing animals were divided into three different groups according to tumor site and size (Table 1). The time intervals between tumor inoculation and measurement of RE function are depicted in Table 1.

The test substance [$^{99}\text{Tc}^m$]-sulfur colloid ($^{99}\text{Tc}^m\text{S}_2$) was prepared according to Persson and Naversten [15]. The range of particle size was 200–500 nm, with a mean of 300 nm, and the number of particles/ml solution was approximately 10^{10} [16]. The preparation was tested in each of the experiments for the labeling efficiency with a gel chromatography column scanning technique [17], and was observed to range between 97 and 100%.

With the animal under general anesthesia with Nembutal 6 mg/kg body wt the jugular vein was cannulated and a fine catheter was introduced and fixed with the tip in the superior vena cava. About 15 MBq in a volume of 0.5 ml containing approximately 0.5×10^{10} particles was injected during 5–10 sec. Registration started immediately at the onset of injection.

After completion of the scintillation camera registration the animals were killed. The liver and spleen were dissected free and extirpated. These organs were then placed on the scintillation camera and measured for total activity content. Each tumor regardless of location was then carefully dissected from surrounding tissue. The weight of the tumor, liver and spleen were recorded.

The kinetics of the activity distribution in the animals were measured with a scintillation camera (Searle, LFOV, Searle Radiographic), equipped with a parallel 39,500 hole collimator. Simultaneous with the start of colloid injection, sequential images were recorded during a 15 min

period. Three frame groups were stored with 16, 12 and 20 frames at 7.5-, 15- and 30-sec intervals respectively. Regions of interest were selected from the total image and time-activity curves were generated. The time-activity curves were stored on an RK06 disc and transferred to a computer (Digital Equipment PDP 11/45), where the uptake curves were evaluated.

An open two-compartment model was used to estimate the flow rates of the radiolabeled colloid to the liver (k_1) and other RE tissues (k_2) (Fig. 1) [14]. Using the same symbols as in Fig. 1, the rate of disappearance of the colloid in the blood (B) and the rate of uptake in the liver (L) with time can be written:

$$\frac{dB}{dt} = -B(k_1 + k_2) \quad (1)$$

$$\frac{dL}{dt} = B k_1 \quad (2)$$

The amount of colloid transported to the liver can then be expressed with the equation:

$$L(t) = 100 \frac{k_1}{k_1 + k_2} [1 - e^{-(k_1 + k_2)t}] \quad (3)$$

The rate constants k_1 and k_2 were obtained by fitting this equation to the experimental uptake curves for each animal [14].

Analysis of the experimental data were performed for 95% confidence with Student's *t* test and results are given as mean values with one standard deviation.

RESULTS

Examination of 8 rats 18–21 days after tumor inoculation revealed in each case the presence of a solitary 0.8- to 2.3-g liver tumor confined to the inoculated lobe (Table 1). These animals had an average weight loss of about 5% of their weight at the time of inoculation.

The rats inoculated with tumor cells subcutaneously had a well-defined 0.3- to 2.8-g subcutaneous tumor nodule 30–36 days after

Table 1. Material presentation, groups of examined animals, No. of tumor cells inoculated, tumor growth time and tumor weight

Group	No.	No. of tumor cells inoculated $\times 10^6$	Time interval (days)	Tumor weight (g)	
				Range	Mean
I. Control	12				
II. Liver tumor	8	1.0	18–21	0.8–2.3	1.6
III. Retroperitoneal tumors	5	0.5	24–28	2.9–20.7	7.0
IV. Subcutaneous tumors	8	1.0	30–36	0.3–2.8	1.3

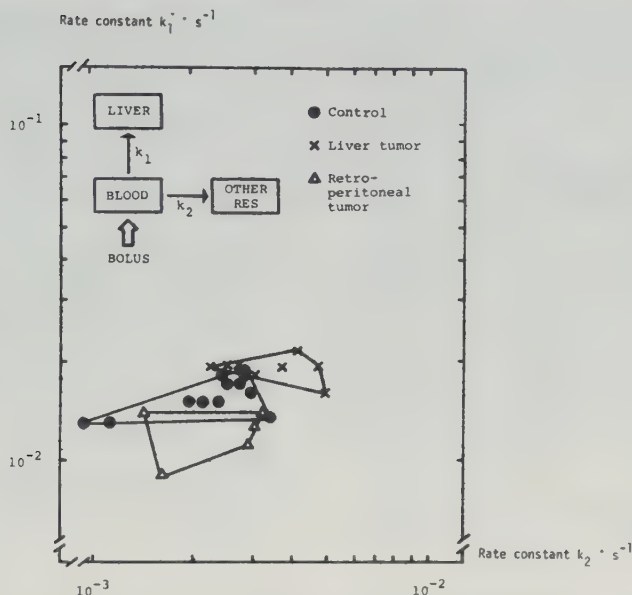


Fig. 1. Scatter diagram of the rate constants k_1 and k_2 obtained from the compartment analysis. Normal rats and rats with liver tumor and retroperitoneal tumor.

inoculation (Table 1). No signs of tumor spread were noted. These animals gained weight during the period from inoculation to examination to the same extent as the control animals.

Examination of the animals with retroperitoneal tumors revealed large tumors (2.9–20.7 g) extending beyond the kidney into the retroperitoneal space. None of the animals showed signs of tumor invasion of the liver or the spleen. These animals had a weight loss during the 24–28 days from inoculation to examination of about 10% (mean value), but no signs of cachexia were apparent (Table 1).

The relative liver weight as a percentage of total

body weight in the 12 control animals was $3.6 \pm 0.7\%$, which was not different from the relative liver weight of tumor-bearing animals (groups II–IV in Table 2). The relative weight of the spleen in the control animals was $0.26 \pm 0.06\%$, whereas for groups II–IV the weight of the spleen was greater (Table 2).

The animals with liver tumors showed a significantly greater uptake rate in the liver (k_1) than the controls ($P < 0.005$) (Table 3). The uptake rate of the extrahepatic RES (k_2) was also significantly higher ($P < 0.005$). Animals with retroperitoneal tumors had a significantly lower k_1 ($P < 0.005$). In this group k_2 values were not

Table 2. Relative weights of liver and spleen (as % of total body weight) and P values in comparison to controls

Groups	Relative weight of liver (%)		Relative weight of spleen (%)	
	Mean $\pm$ S.D.	$P <$	Mean $\pm$ S.D.	$P <$
I. Controls	3.6 ± 0.7		0.26 ± 0.06	
II. Liver tumors	3.7 ± 0.3	n.s.*	1.10 ± 0.06	0.001
III. Retroperitoneal tumors	3.4 ± 0.5	n.s.	0.76 ± 0.40	0.001
IV. Subcutaneous tumors	3.6 ± 0.5	n.s.	0.60 ± 0.09	0.001

*n.s. = No significance.

Table 3. [$^{99}\text{Tc}^m$]-sulfur colloid uptake rate and total organ uptake in % of given activity and P values compared to controls

Groups	Colloid uptake rate (sec^{-1})				Total organ uptake			
	Liver $k_1 \times 10^{-2}$	P <	Spleen $k_2 \times 10^{-3}$	P <	Liver (%)	P <	Spleen (%)	P <
I. Controls	1.6 ± 0.2		2.2 ± 0.7		89 ± 3		3.2 ± 1.5	
II. Liver tumors	1.9 ± 0.1	0.005	3.5 ± 0.9	0.005	84 ± 3	0.005	6.4 ± 1.5	0.001
III. Retroperitoneal tumors	1.2 ± 0.2	0.005	2.4 ± 0.8	n.s.	84 ± 3	0.01	7.6 ± 2.1	0.02
IV. Subcutaneous tumors	1.8 ± 0.3	0.05	4.5 ± 1.7	0.005	81 ± 8	0.01	5.7 ± 1.9	0.005

different from those of the control group. Rats with subcutaneous tumors showed a significantly higher k_1 ($P < 0.05$) and k_2 ($P < 0.005$). k_1 and k_2 values for normal and tumor-bearing rats are presented as scatter diagrams in Figs 1 and 2. For control rats the total activity uptake was $89 \pm 3\%$ for the liver and $3.2 \pm 1.5\%$ for the spleen. Total activity uptake by the liver was lower in all tumor animals. Total activity uptake by the spleen was higher in all tumor-bearing animals (Table 3).

DISCUSSION

Previous clearance studies of the reticuloendothelial function in relation to experimental tumor

growth have not discriminated between different compartments of the stationary RE system. These studies have shown an influence of the total RE phagocytic activity related to tumor size [2, 3]. The relationship between RE function and tumor localization has not been elucidated.

In the present study tumors of similar size, located in the liver or dorsal subcutaneous tissue, independent of localization, caused an increased colloid uptake rate in the liver as well as in the spleen.

A correlation between uptake rate and total organ uptake was not invariably seen. Colloid uptake rate may reflect the functioning status of

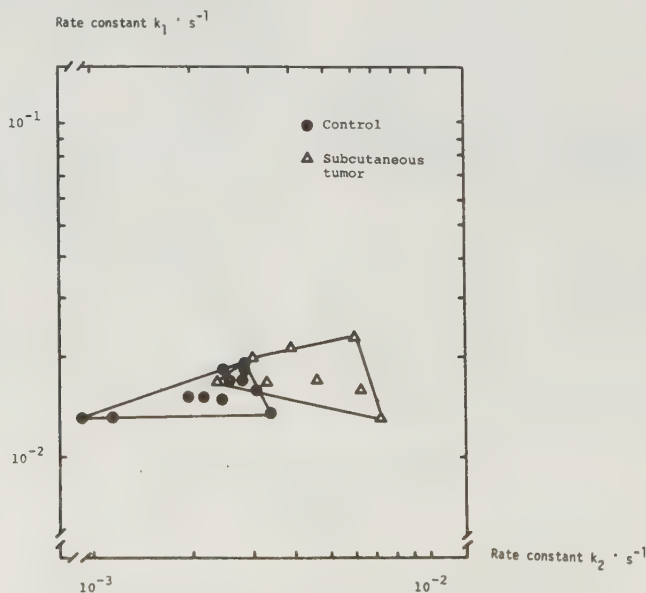


Fig. 2. Scatter diagram of the rate constants k_1 and k_2 obtained from the compartment analysis. Normal rats and rats with subcutaneous tumors.

the RE system and is influenced by factors such as opsonins. The total organ uptake reflects more the total number of functioning macrophages [6].

The total uptake of the spleen was increased to the same degree in both the group with liver tumors and the group with subcutaneous tumors. The high values of total splenic colloid uptake rate coincided with a higher relative weight of the spleen and with an increased total organ uptake of the spleen in these two groups. An increase in the size of the spleen in animals with experimental tumor growth has been described earlier, and has been attributed to an increased number of RE cells [2]. The weight of the spleen in animals with liver tumors was about double that of animals with subcutaneous tumors of similar size. An obvious explanation for this difference cannot be postulated. Since the liver tumors were all located in the periphery of the liver and did not cause any portal stasis, portal hypersplenism is not likely to be the cause of the difference.

The total organ uptake of the colloid by the liver was lower in tumor animals, regardless of tumor localization. However, no changes in the relative weights of the livers in tumor-bearing animals were seen. The lower percentage total uptake in the liver may be explained by the higher uptake of the enlarged spleen, as well as a higher uptake in the remaining parts of the RES.

In animals with large retroperitoneal tumors colloid uptake rate in the liver was significantly decreased. The total organ uptake of the colloid in the spleen and the splenic weight was also higher in this group, although the uptake rate was unchanged compared to the controls. These large tumors may cause an exhaustion of RE function, perhaps through a reduction of opsonin factors. This corresponds to earlier findings of a depression of RE function in animals with large

experimental tumors [2]. The uptake rate of the extrahepatic RES was, however, significantly decreased compared to animals with smaller tumors (groups II and IV). This may reflect that the suppression of RE function with increasing tumor burden is seen earlier in the hepatic RES than in the extrahepatic RES.

The RES phagocytic capacity can be measured by the clearance of colloidal particles from the blood stream [2, 6, 12]. [$^{99}\text{Tc}^m$]-sulfur colloid has been used extensively in static liver scanning, as well as for studies of the reticuloendothelial phagocytic function [6, 13]. In contrast to previous methods, the technique used in the present study [14] enables separation of the hepatic and the extrahepatic RES. The spleen is by far the most important extrahepatic RE organ [6]. The present results indicate that the influence of tumor growth on the RES is mainly a function of tumor size. A varied influence of hepatic and extrahepatic RES from various tumor burdens was observed. This finding indicates the value of distinguishing between various compartments of the RES.

The present technique provides a simple and reliable method of studying RE function in the experimental animal. The good reproducibility found in earlier studies has been further confirmed in this present study.

A clinical implication of a stimulated RE system during tumor growth may be an increased uptake by the RES of monoclonal antibodies used for diagnostic purposes. Suppression of such RES uptake by pharmacological means may enhance the diagnostic efficiency of such antibodies.

Further research is necessary to determine if a lower RE function in the liver as measured by the colloid uptake rate has any clinical implication in the treatment of liver cancer.

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RELEASE OF β -HEXOSAMINIDASE AFTER ADMINISTRATION OF DIFFERENT AGENTS AFFECTING THE RETICULOENDOTHELIAL SYSTEM. AN EXPERIMENTAL STUDY IN RATS.

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ABSTRACT

Plasma levels of a lysosomal enzyme, β -hexosaminidase (β -N-acetylglucosaminidase, E.C. 3.2.1.30) were studied in Wistar rats after administration of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid, ^{198}Au -colloid, gelatine (Haemacel[®]), alcohol, methyl palmitate and zymosan. The activity of β -hexosaminidase was increased 10, 30 and 60 minutes after the zymosan injection. After 24 and 48 hours, enzyme levels had returned to those at outset. The transient release of β -hexosaminidase probably occurred only during the phagocytosis of zymosan which was evaluated by histological examination of lung, liver and spleen. After the injection of all other agents tested, no significant aberration of β -hexosaminidase levels was seen. Activity distribution of the radiolabeled colloids revealed differences in organ uptake which were attributed to a difference in colloid particle size. Although the colloids tested have been used extensively for determination of reticuloendothelial function and histological studies suggest phagocytosis of the particles, their administration did not affect plasma β -hexosaminidase levels. Since lysosomal enzymes are cleared from the blood predominantly by liver macrophages, the primary location of particle phagocytosis may explain the present findings.

KEY WORDS: β -hexosaminidase; zymosan; radiolabeled colloids; Kupffer cells; macrophages; lysosomal enzymes.

INTRODUCTION

Several methods are available for measurement of reticuloendothelial (RE) function (6, 8, 33). These methods are based upon studies of the fate of injected colloidal particles. Colloidal preparations with different properties have been used for this purpose. Differences in the rate of particle extraction by the reticuloendothelial system (RES) have been attributed to variations in the size, number and surface charge of injected particles (33, 40).

Morphological examinations have also revealed differences in the mechanism of particle uptake by macrophages (9, 16). In earlier studies of RE function, we have used a highly standardized preparation of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid (28, 31, 32). ^{198}Au colloid has also been used as an agent for studies of the RES (9, 40). In a comparative study of the localization of injected $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and ^{198}Au colloid, only the ^{198}Au colloid was retrieved intracellularly in Kupffer cells (9). George et al (16) also failed to show ingestion of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloidal particles in Kupffer cells. However, histological and ultrastructural studies in our laboratory have shown phagocytosis of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid (31). The stimulatory effect on the RES of zymosan has been explored by numerous investigators (12, 13, 29, 30, 41, 46). Histological examinations have revealed that zymosan is phagocytized by macrophages (19, 41).

The reticuloendothelial depressant effect of methyl palmitate has been thoroughly evaluated (7, 14, 34). Histological evidence has indicated that the RE depressant effect of methyl palmitate is not due to destruction of RE cells but rather to an interference with the phagocytic activity of Kupffer cells (14). Gelatine (Haemaccel[®]) has also been shown to impair RE phagocytic function (31, 36).

Studies of the intracellular distribution of lysosomal enzymes in liver macrophages have shown that these enzymes are increased in elicited macrophages (18). Secretion of lysosomal enzymes from macrophages has been observed during the process of phagocytosis (10, 11, 27, 44, 45). The release of β -hexosaminidase during phagocytosis in vitro of opsonized latex particles was more pronounced from monocyte derived macrophages than from peripheral blood monocytes (25). These investigators have also observed release of β -hexosaminidase by cultured macrophages in response to serum opsonized zymosan. In addition to the secretory capacity of lysosomal enzymes by macrophages, recent observations have identified the importance of macrophages in the blood clearance of these enzymes (1, 37, 42).

Increased levels of lysosomal enzymes have been correlated to depression of RE function after experimental trauma (23). A decreased Kupffer cell function has also been demonstrated after acute and chronic alcohol administration to animals (2, 26). Consequently, elevation of serum levels of β -hexosaminidase was found in patients

with various diseases of the liver and in patients with acute ethanol intoxication (20, 21).

The principal aim of the present study was to investigate the effect on the plasma levels of β -hexosaminidase of substances known to affect RE function (zymosan, methyl palmitate, gelatine (Haemacel[®]) and alcohol) or used as test substances of RE function ($^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and ^{198}Au colloid).

The organ distribution of the two colloids was investigated in order to reveal possible differences in localization of the two radiolabeled preparations.

Morphological studies were carried out after injection of ^{198}Au colloid and compared with previous results regarding the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid. Histological examinations regarding the localization of injected zymosan were also performed.

MATERIALS AND METHODS

Male Wistar rats, 150-205 g, were used in all experiments. For the study of enzyme levels, 41 rats were divided into 7 groups according to the administered agent (Table I). Under ether anesthesia the jugular vein was cannulated and the agents were administered intravenously.

Table I. Design of the study. Types and amount of administered agents.

Groups	Administered agent	Concentration	Amount
A	Saline (control)	0.9 %	2 ml
B	$^{99}\text{Tc}^{\text{m}}$ -sulphur colloid	12 mg $\text{Na}_2\text{S}_2\text{O}_3$	1 ml *
C	^{198}Au -colloid	5.2 mg Au/ml	1 ml *
D	Gelatine (Haemacel [®])	3.5 %	2 ml
E	Alcohol	96 %	0.25 ml *
F	Methyl palmitate	400 mg/ml	250 mg/100 g *
G	Zymosan	10 mg/ml	3 mg/100 g *

* Diluted with saline to a volume of 2 ml.

The $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid was prepared as described earlier (28, 31). The mean particle size was 300 nm (5). A standard preparation of colloidal gold (^{198}Au) with a particle size of 5-20 nm was used

(Amersham Radiopharmaceuticals, England). Gelatine was administered as Haemaccel® (Behring). Alcohol was administered as 0.25 ml 96 % alcohol in 2 ml saline giving a calculated blood concentration of about 1.3 o/oo. Methyl palmitate (Sigma Co) was prepared as an emulsion by sonification for 10 min in 5 % dextrose solution and 0.1 % Tween 20 (14). Zymosan (Sigma Co) was suspended in saline in a concentration of 10 mg/ml (41). Concentrations and injected amounts are given in Table I.

Heparin-plasma samples (0.5 ml) were collected from the catheter before and 10, 30 and 60 min after administration of the different substances. Plasma samples were collected from the tail vein after a lapse of 24 and 48 hours. All samples were analysed for plasma activity of β -hexosaminidase with an earlier described method (20). The coefficient of variation for the method was below 10 %.

For the study of the distribution of the radiolabeled colloids, 4 rats received the $^{99}\text{Tc}^m$ -sulphur colloid and 5 rats were given the ^{198}Au colloid. With the animal under Nembutal anesthesia and fixed on a scintillation camera, 0.5 ml of the test colloid was injected through a catheter in the jugular vein. With the scintillation camera (Searle, LFOV, Searle Radiographic Inc), equipped with a parallel 39 500 hole collimator, the accumulation of activity in the liver and spleen was registered during 15 minutes after injection whereafter the liver uptake curves reached a plateau. After this, all animals were sacrificed. A blood sample was collected. For measurements of organ activity, the right lung and the right kidney and a muscle sample from the thigh were carefully dissected. The liver and the spleen were extirpated and a bone marrow aspirate was collected from both femurs with fine needle aspiration. Each organ or tissue was inserted into a plastic tube, weighed and measured for radioactivity with an automatic sample-changing NaI (Tl) well counter.

For morphological studies of the uptake of ^{198}Au colloid, liver and spleen specimens were collected from 8 rats, sacrificed at 1, 3, 5, 10, 30 and 60 minutes and 24 and 48 hours, respectively, after administration of 0.5 ml ^{198}Au colloid suspension. Slices were fixed in 4 % formaldehyde, embedded in paraffin and stained with hematoxylin-erytrosine, van Gieson, the Turnbull-blue reaction for iron and with Fouchet's reagent for bilirubin pigment.

Twenty-eight rats were used for histological examination of the lung, the liver and the spleen after zymosan injection. Zymosan was prepared as previously described. Under ether anesthesia 16 rats received an intravenous injection of zymosan (3 mg/100 g) and 12 rats were injected with an equal amount of saline. All injections were made through a jugular catheter. Four zymosan-injected animals and three control animals were sacrificed at each of the following intervals after the injections: 20, 60 and 120 min and 24 h. Specimens from lung, liver and spleen were collected, prepared as described above,

and stained with hematoxylin-erythrosine, PAS and PAS preceded by diastase treatment.

When testing for differences, Student's t-test was used. The significance was also verified with the non-parametric Wilcoxon rank sum test. A p-value of less than 0.05 was considered significant.

RESULTS

The changes in enzyme activity in the different groups compared with basal enzyme levels are given in Table II. β -hexosaminidase levels were significantly raised over basal levels, at 10 and 30 minutes ($p < 0.05$) and at 60 min ($p < 0.001$) after the injection of zymosan. No difference in enzyme activity was observed between 10 and 30 minutes, however, a statistically significant ($p < 0.025$) increase was observed between 30 and 60 minutes. Twenty-four and 48 hours after injection of zymosan, the β -hexosaminidase activity had returned to basal levels. Enzyme activity was, in comparison with that of the controls, increased at 10 min ($p < 0.05$), 30 and 60 min ($p < 0.001$) in zymosan injected animals (Fig 1).

Table II. Relative activity $\pm$ SEM of β -hexosaminidase 10, 30 and 60 min after injection of test substance, p-values in comparison to basal levels.

Groups	Administered agent	Animals (n)	Basal	10 min	p <	30 min	p <	60 min	p <
A	Control (NaCl)	8	1.00 $\pm$ 0.07	1.04 $\pm$ 0.05		0.86 $\pm$ 0.03		0.89 $\pm$ 0.06	
B	$^{99}\text{Tc}^{\text{m}}$ -sulphur colloid	5	1.00 $\pm$ 0.08	1.09 $\pm$ 0.06		0.97 $\pm$ 0.07		0.88 $\pm$ 0.06	
C	^{198}Au -colloid	5	1.00 $\pm$ 0.08	0.74 $\pm$ 0.19		0.83 $\pm$ 0.20		0.84 $\pm$ 0.08	
D	Gelatin (Haemacel [®])	5	1.00 $\pm$ 0.08	0.86 $\pm$ 0.05		1.01 $\pm$ 0.10		1.05 $\pm$ 0.03	
D	Alcohol	6	1.00 $\pm$ 0.27	0.92 $\pm$ 0.19		1.08 $\pm$ 0.13		1.06 $\pm$ 0.11	
F	Methyl palmitate	7	1.00 $\pm$ 0.12	0.92 $\pm$ 0.08		1.02 $\pm$ 0.11		1.10 $\pm$ 0.14	
G	Zymosan	5	1.00 $\pm$ 0.09	1.36 $\pm$ 0.07	0.05	1.35 $\pm$ 0.06	0.05	1.63 $\pm$ 0.05	0.001

For all other agents tested, no statistically significant variation in β -hexosaminidase activity was found when compared with basal levels in each group or compared with controls at corresponding intervals after injection (Table II). No differences in enzyme levels were noted 24 and 48 hours after injection.

The total accumulated activities in the liver and the spleen 15 minutes after the injection of the radiolabeled colloids are depicted in Table III. The uptake of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid was significantly higher in the liver and the spleen than the corresponding uptake of the ^{198}Au colloid.

Change in plasma β -hexosaminidase %

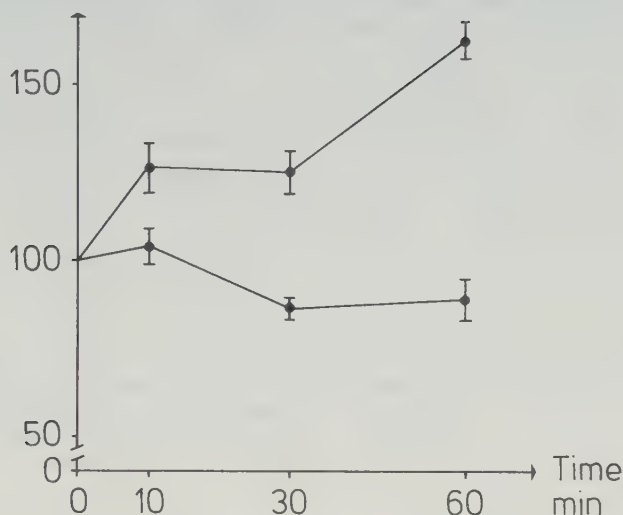


Fig 1. Change in β -hexosaminidase activity (mean $\pm$ SEM) after zymosan injection (upper curve) compared to control (lower curve).

Table III. Organ weight (mean $\pm$ SD) and total activity uptake in liver and spleen of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and ^{198}Au colloid, percentage of injected dose (mean $\pm$ SD). p-values for comparison of differences according to Student's t-test (n.s. = not significant at the 0.05 level).

	n	Organ weight (gram)		Accumulated activity (%)	
		Liver	Spleen	Liver	Spleen
$^{99}\text{Tc}^{\text{m}}$ -S-colloid	4	7.0 $\pm$ 1.0	0.51 $\pm$ 0.02	92.6 $\pm$ 2.2	3.4 $\pm$ 0.8
^{198}Au	5	6.1 $\pm$ 1.0	0.54 $\pm$ 0.06	85.3 $\pm$ 3.2	1.9 $\pm$ 0.2
p		n.s.	n.s.	< 0.01	0.01

The distribution of the radiolabeled colloids in various organs and tissues in percentage of injected activity per g is depicted in Table IV. When measuring the extirpated organs, the difference in total splenic uptake was verified. Significant differences were also registered regarding the uptake of the two colloids in the lung and in the bone marrow.

Table IV. Activity distribution in percent of injected activity per gram tissue (mean $\pm$ SD) in different organs for $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid and ^{198}Au colloid. p-values for comparison of differences according to Student's t-test.

	Blood	Liver	Spleen	Lung	Bone marrow	Kidney	Muscle
$^{99}\text{Tc}^{\text{m}}\text{-S}$	0.066 $\pm$ 0.051	13.4 $\pm$ 1.6	6.5 $\pm$ 1.1	0.47 $\pm$ 0.24	0.18 $\pm$ 0.04	0.055 $\pm$ 0.005	0.0018 $\pm$ 0.0004
^{198}Au	0.15 $\pm$ 0.07	14.2 $\pm$ 2.0	3.6 $\pm$ 0.4	0.11 $\pm$ 0.02	2.8 $\pm$ 0.5	0.047 $\pm$ 0.014	0.0028 $\pm$ 0.009
p	n.s.	n.s.	< 0.001	< 0.02	< 0.001	n.s.	n.s.

Light microscopical examination after ^{198}Au colloid administration demonstrated vacuoles in the hepatocytes, especially in the central parts of the lobuli from 1 to 60 minutes after injection. Vacuoles were also seen in sinusoidal cells. No changes in the liver were detected after 24 and 48 hours. Examination of the spleens revealed no changes at any time.

Histological examination of lung, liver and spleen after different intervals following zymosan injection revealed no changes in the spleens.

In the livers, the sinusoidal cells in the peripheral parts of the lobuli were larger and contained more PAS-positive, diastase-resistant material at 1 and 2 h than at 20 min and 24 h. At all times there was very little of such material in the controls. In the areas with prominent sinusoidal cells, globules with this staining characteristic were also found in the liver parenchymal cells and considerably more than at 20 min and 24 h (Fig 2 c). There were almost no such globules in the controls.

Small granulomas of sinusoidal cells were found at 2 h (Fig 2 b). At 24 h there were several granulomas, containing neutrophile polymorphonuclear leukocytes and sinusoidal cells in varying proportions, especially in the peripheral parts of the lobuli and also bordering to the portal tracts (Fig 2 a). There may have been necrosis of parenchymal cells in these granulomas. There were no granulomas in the controls.

In the lungs a conspicuous amount of neutrophils were found in the interstitial tissue and/or in the small blood vessels at 20 min and 1 and 2 h often in small heaps (Fig 2 d). There were a few granuloma-like structures at 2 h (Fig 3 a). At 24 h the inner layer of the small

blood vessels were often considerably thickened by cells, and neutrophils were often found around the vessels and in larger number than in the controls (Fig 3 b). A few granuloma-like areas were also encountered.

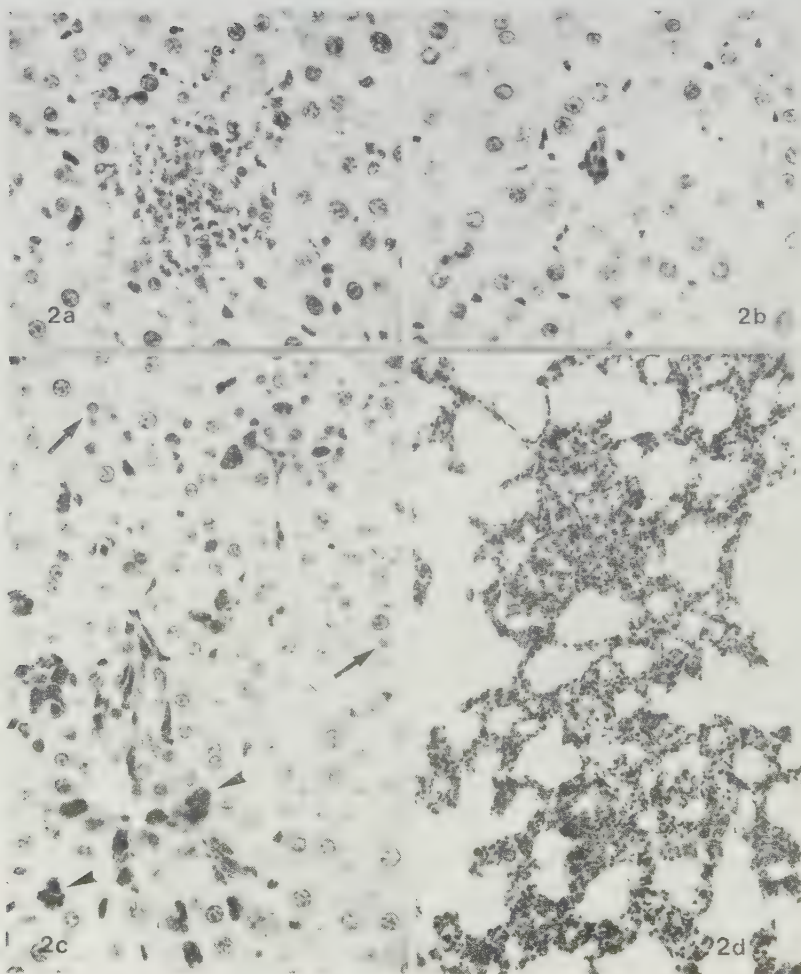
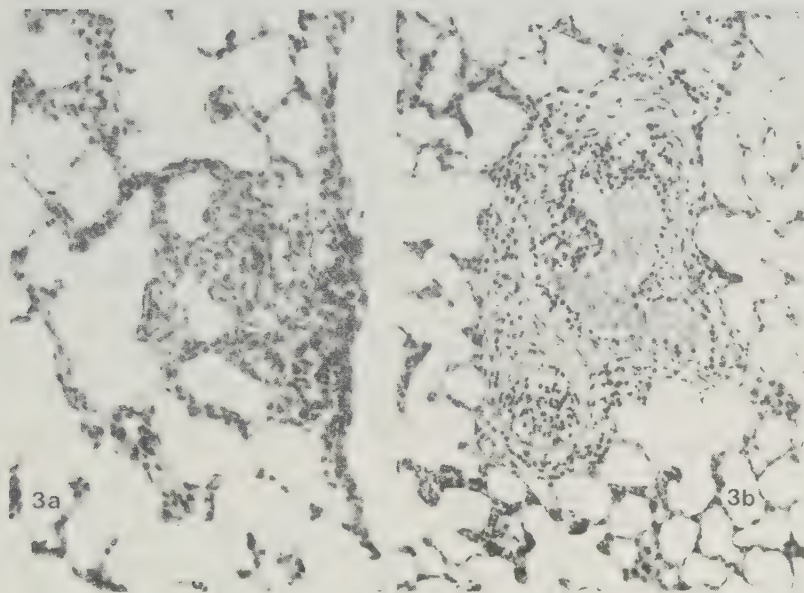


Fig 2. a) Large granuloma in the liver containing both granulocytes and Kupffer-like cells 24 h after zymosan injection. Hematoxylin-erythrosine x 400. b) Small granuloma of probably Kupffer cells in the liver 2 h after administration of zymosan. Hematoxylin-erythrosine x 400. c) Prominent Kupffer cells (delta-arrows) in the peripheral part of a liver lobulus 2 h after zymosan injection. Globules of varying size are found in several parenchymal cells (arrows). PAS-staining after treatment with diastase. x 165. d) Section of lung 1 h after administration of zymosan. Granulocytes are often found in large amount in the tissue. Hematoxylin-erythrosine x 165.

Fig 3. a) A granuloma in the peripheral part of the lung 24 h after zymosan injection. Hematoxylin-erythrosine x 250. b) Blood vessel with thickened intima in the lung 24 h after administration of zymosan. Granulocytes are found perivascularly. Hematoxylin-erythrosine x 165.



DISCUSSION

Zymosan, a yeast product, derived from the cell wall of *Saccharomyces Cerevisiae* has been demonstrated to produce marked hyperplasia and hyperfunction of the RES (4, 29, 30). The active RE stimulant component of zymosan is glucan, a β -1,3-polyglucose (29). Endocytosis of zymosan has been shown to be very rapid and morphological studies have revealed that this occurs primarily in lung macrophages but zymosan is later found in Kupffer cells (41). The present results of the histological examinations of lung and liver suggest that the yeast particles are taken up by macrophages of the lung and liver. The uptake in the lung seems to precede liver uptake which is in consistency with an earlier study by Sieck et al (41). The globules apparent in the preparations from the liver probably represent yeast particles. The typical granuloma formation in the liver seen after zymosan administration was apparent at 2 h after injection but was most prominent at 24 h after injection. This observation is in agreement with previous results (13, 14, 29, 41).

Treatment in vitro of cultured resident macrophages with zymosan causes a rapid extracellular release of acid hydrolasis and a corresponding decrease in intracellular enzyme levels both of which returned to normal after several days (18). Cultured human macrophages, derived from peripheral blood monocytes have been shown to release β -hexosaminidase in response to serum opsonized zymosan (25). After the injection of zymosan to rats, serum activity of certain lysosomal enzymes such as β -glucuronidase and acid phosphatase have been found to increase, but return to normal levels after 2 hours (41). In the present study similar findings were obtained concerning another lysosomal enzyme, β -hexosaminidase. The increase of plasma β -hexosaminidase level is only transient and seems to exist solely during the phagocytic process of zymosan. The two-step rise in β -hexosaminidase activity after zymosan injection may be due to the fact that zymosan is first endocytosed by lung macrophages and later by Kupffer cells, as suggested by the histological examination. Turn-over of rat tissue β -hexosaminidase is known to be short (0.4 days) (37). This may also explain why the rise in enzyme activity is of short duration.

The two different colloids used in the present study have been used extensively as radiolabeled detectors of macrophage function (8, 9, 31, 33). The histological examination following ¹⁹⁸Au colloid injection suggests phagocytosis of the particles in hepatocytes and RE cells of the liver. Similar results have been obtained concerning the ⁹⁹Tc_m-sulphur colloid. These histological findings were also confirmed by ultrastructural studies (31). However, autoradiographic studies by others have failed to demonstrate radioactive material intracellularly (9). Ultrastructural studies of the uptake of sulphur colloid by hepatic macrophages demonstrated the particles in clusters on the surface of Kupffer cells but not intracellularly (16). These diffe-

rences may be explained by different colloid preparations, since differences in colloid particle size may influence the organ uptake (3, 40).

In a comparative study of ^{198}Au and $^{99}\text{Tc}^{\text{m}}$ -sulphur colloids Mundschenk et al observed that the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid was accumulated in the spleen to a greater extent than the ^{198}Au colloid (24). Using preparations of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloids with different particle size, smaller particles accumulated comparatively more in the bone marrow than particles of a greater size (3, 35). In the present study, the ^{198}Au colloid accumulated to a greater extent in the bone marrow than the $^{99}\text{Tc}^{\text{m}}$ -colloid. Although the accumulated activity in the liver of the $^{99}\text{Tc}^{\text{m}}$ -colloid was higher than that of the ^{198}Au -colloid no difference was observed when comparing the organ activity in per cent per gram. Variations in liver weight between the two groups may explain this observation. The significant differences in the uptake in the spleen and the bone marrow, observed in the present study, are probably due to the difference in colloid size. This may also be the explanation for the greater uptake in the lungs of $^{99}\text{Tc}^{\text{m}}$ -sulphur particles since the injected particles first pass the lung capillaries. The organ distribution of the $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid found here is also in good agreement with that reported by Frier et al (15).

A variety of particulate and soluble stimuli from chronic inflammation trigger synthesis in and release of lysosomal enzymes from macrophages (22, 38, 39). The intravenous administration of certain colloidal and macromolecular substances to animals causes increased levels of acid hydrolase in the liver (17, 43). In the present study, none of the injected colloids caused significant alterations in plasma levels of β -hexosaminidase. This may be explained by the fact that most particles, during the time of study (60 min), merely adhere to the surface of phagocytes (16). Another possible explanation is that the released enzyme is immediately cleared from the blood by neighboring Kupffer cells and never reach the systemic circulation, in contrast to enzyme released from other organs as the lungs (37, 42).

Alcohol depresses phagocytic activity as measured by the clearance of microaggregated albumin (2). Acute and chronic administration of alcohol has been associated with delayed endotoxin clearance in rats which was related to the dose of alcohol (26). Although increased levels of β -hexosaminidase were found in chronic alcoholics with acute alcohol intoxication (21), alcohol may not be the only responsible factor for this observation. The dose of alcohol may also influence the levels of enzyme release. In the present study no increase in β -hexosaminidase activity was found after administration of a single dose of alcohol which may be explained by the fact that the dose was too low or by the absence of an underlying hepatic injury.

Methyl palmitate and gelatine (Haemaccel[®]) have been shown to impair phagocytic activity (14, 31, 36). No changes in hepatic morphology or

liver cell number have been observed following methyl palmitate administration (14). The phagocytic depression following methyl palmitate injection is primarily due to a selective hepatic and splenic macrophage impairment (34). However, none of these RE depressant agents influenced β -hexosaminidase activity in the present study. This may suggest that the clearance of β -hexosaminidase was not altered by these substances.

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INFLUENCE ON PLASMA β -HEXOSAMINIDASE OF RETICULOENDOTHELIAL STIMULATION AND DEPRESSION. An experimental study in rats.

Running title: Plasma β -hexosaminidase and RES function.

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Key words: β -hexosaminidase, methyl palmitate, zymosan, macrophages, Kupffer cells.

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SUMMARY

Plasma levels of the lysosomal enzyme, β -hexosaminidase (β -N-acetylglucosaminidase; EC 3.2.1.30; 2-acetoamido-2-deoxy- β -D-glucoside acetamidodeoxyglucohydrolase) were estimated 120 minutes after intravenous injection of zymosan in rats. Different groups of animals were investigated according to pretreatment with agents influencing reticuloendothelial activity. Pretreatment for two days with the RE stimulating agent, zymosan, or the RE suppressing agent, methyl palmitate, did not influence the basal plasma levels of β -hexosaminidase.

In all groups, plasma β -hexosaminidase activity was increased compared to basal levels after zymosan injection. The increase of enzyme activity was most pronounced after pretreatment with zymosan and differed significantly from enzyme activity after pretreatment with methyl palmitate. Alcohol administration in combination with zymosan did not cause a further rise of β -hexosaminidase activity in normal rats nor in rats pretreated with zymosan.

The observations suggest that plasma levels of β -hexosaminidase after a standardized zymosan injection to rats are related to the functional status of the RES.

INTRODUCTION

An increased serum level of β -hexosaminidase has been found in patients with different diseases of the liver and in patients with acute ethanol intoxication (1, 2). The mechanisms leading to the elevated level of lysosomal enzymes are not clear. One possible reason could be an activation of body macrophages leading to an increased synthesis and secretion of lysosomal enzymes. Another reason could be a defect clearance from the blood of lysosomal enzymes, due to a decreased Kupffer cell function.

It has been proposed that serum levels of lysosomal enzymes could serve as markers for macrophage activation in mice (3). The release of lysosomal enzymes has been shown to be correlated with the process of phagocytosis (4).

Zymosan, a yeast product derived from the cell wall of *Saccharomyces Cerevisiae*, has been demonstrated to activate macrophages (5) and to produce a marked hyperplasia and hyperfunction of the reticuloendothelial system (6, 7). In a previous study an elevation of the lysosomal enzyme, β -hexosaminidase, was found in serum after injection of a single dose of zymosan in the rat (Rydén S, unpublished data 1983). Methyl palmitate is a well-known suppressor of the reticuloendothelial phagocytic activity (8).

The aim of the present study was to investigate the influence of reticuloendothelial zymosan-stimulation and methyl palmitate-depression on the activity of β -hexosaminidase in plasma after a single dose of zymosan. The possibility that alcohol could influence the release of β -hexosaminidase was also studied.

MATERIAL AND METHOD

The effect of reticuloendothelial stimulation or depression was studied in 25 male Wistar rats, weighing 154-192 g. The animals were divided into five groups according to the administered agents (Table 1). All animals were fed on standard food pellets and water ad libitum.

Table 1. Design of study. Time schedule of administered agents and administered doses.

Group	Number	Days 1 and 2		Day 3			
		Agent 1	Dose	Agent 2	Dose	Agent 3	Dose
A	5	-	-	-	-	Zymosan	3 mg/100 g
B	5	Zymosan	3 mg/100 g	-	-	Zymosan	3 mg/100 g
C	5	Methyl palmitate	200 mg/100 g	-	-	Zymosan	3 mg/100 g
D	5	Zymosan	3 mg/100 g	Alcohol 96 %	0.25 ml	Zymosan	3 mg/100 g
E	5	-	-	Alcohol 96 %	0.25 ml	Zymosan	3 mg/100 g

Methyl palmitate (Sigma Co.) was prepared as an emulsion by sonification for 10 min in 5 % dextrose and 0.1 % Tween 20 at a concentration of 400 mg/ml.

Zymosan (Sigma Co.) was suspended in 0.9 % saline at a concentration of 10 mg/ml.

Pretreatment was given on two consecutive days with zymosan (groups B and D) and methyl palmitate (group C) by intravenous injection into the jugular vein with the animal under light ether anesthesia.

On the day of the experimental procedure, the jugular vein was cannulated and heparine-plasma samples for basal levels of β -hexosaminidase were collected. Zymosan was injected into all the animals and blood sampling (0.5 ml) was carried out 10, 30, 60, 90 and 120 min after the zymosan injection. This injection was preceded by an intravenous injection of alcohol in groups D and E (Table 1). Plasma was analyzed for β -hexosaminidase activity using a method described elsewhere (1).

Results are given as mean $\pm$ SEM. Statistical analyses for 95 % confidence were performed with Student's t-test and the non-parametric Wilcoxon rank sum test.

RESULTS

β -hexosaminidase levels for the different groups are depicted in Table 2. After the zymosan injection a statistically significant increase of β -hexosaminidase levels was seen in all groups. No deviation of basal levels of β -hexosaminidase was found in animals given pretreatment with zymosan (B and D) or methyl palmitate (C) when compared with the non-pretreated groups (A and E).

Table II. Activity of β -hexosaminidase in serum before (basal) and after zymosan injection. Statistical significance for comparison to basal activity in each group with Student's t-test: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Group	β -hexosaminidase activity					
	Basal	10	30	60	90	120 minutes after zymosan injection
A	12.0 $\pm$ 0.6	12.7 $\pm$ 0.7	17.7 $\pm$ 0.6*	18.1 $\pm$ 0.7***	16.7 $\pm$ 1.2**	17.0 $\pm$ 0.4**
B	11.5 $\pm$ 0.7	14.5 $\pm$ 0.6**	18.7 $\pm$ 1.2**	21.1 $\pm$ 2.2**	21.7 $\pm$ 2.4**	18.7 $\pm$ 1.3**
C	11.4 $\pm$ 0.7	12.4 $\pm$ 1.4	15.3 $\pm$ 0.4*	15.7 $\pm$ 1.1*	16.4 $\pm$ 1.2**	15.3 $\pm$ 1.3**
D	11.8 $\pm$ 0.2	15.8 $\pm$ 1.3*	19.2 $\pm$ 2.0*	18.9 $\pm$ 2.1*	18.1 $\pm$ 1.3**	19.1 $\pm$ 1.8*
E	12.9 $\pm$ 1.0	15.2 $\pm$ 0.5*	19.7 $\pm$ 1.0**	22.9 $\pm$ 1.6**	22.6 $\pm$ 1.4**	20.5 $\pm$ 1.6**

After 60 min, the level of β -hexosaminidase was significantly higher in the zymosan pretreated rats (B) than in the methyl palmitate pretreated rats (C) with a p value of 0.05 (Fig 1). After 90 min, the enzyme level of the zymosan pretreated rats was significantly higher than that in the control (A) and the methyl palmitate pretreated rats (C) with p values of 0.05 in both comparisons (Fig 1).

Betahexos- aminidase (U/l)

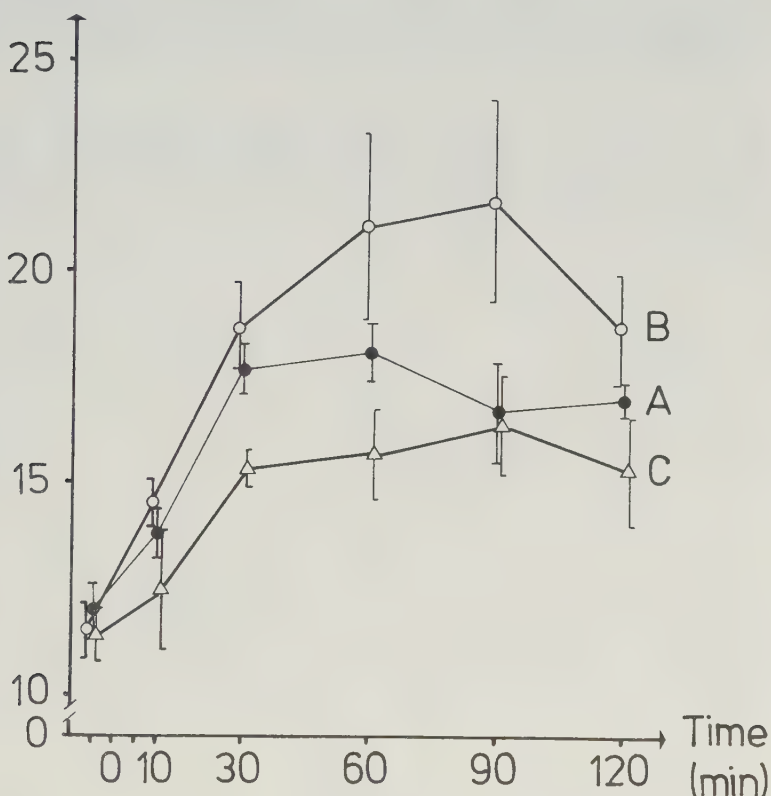


Fig 1. β -hexosaminidase activity after zymosan injection into control animals (A), into animals pretreated with zymosan (B) and methyl palmitate (C).

The administration of alcohol (D and E) did not significantly influence the rise of β -hexosaminidase activity when compared with the corresponding groups (A and B) where alcohol was not given.

DISCUSSION

Phagocytizable and digestable particles such as sheep red blood cells or aggregated gammaglobulin are powerful stimulants of intracellular hydrolase activity but cause little extracellular enzyme release (9, 10). On the other hand, in vitro treatment of cultured resident mouse macrophages with streptococcal cell walls (11) causes a rapid extracellular release of performed tissue acid hydrolases and a corresponding decrease in intracellular enzyme levels. Zymosan-elicited rat peritoneal macrophages exhibited little change in intracellular levels of glucuronidase, galactosidase and acid phosphatase but elevated levels of glucosaminidase (12).

Zymosan given as a single intravenous dose has been shown to increase the RE function in rats for at least 72 hours (7). The active RE stimulant component of zymosan is glucan, a β -1,3-polyglucos (7). Repeated administration of glucan to rats has shown to produce a marked hyperplasia of Kupffer cells and mononuclear granuloma formation in the liver (6, 7, 13).

Serum activity of certain lysosomal enzymes such as β -glucuronidase and acid phosphatase has been shown to be considerably increased as soon as 20 min after the injection of zymosan in rats (14). Human macrophages derived from peripheral blood monocytes, cultured in vitro, have been shown to release β -hexosaminidase in response to serum-opsonized zymosan (15). Thus zymosan is a powerful substance for the release of lysosomal enzymes from macrophages. The increase of serum lysosomal enzyme levels is, however, only transient and seems to exist only during the phagocytic process of zymosan (14). The rapid release of lysosomal enzymes after zymosan injection probably results from the release of stored lysosomal enzymes (tissue form). Turnover of rat β -hexosaminidase is known to be very short when purified preparations (liver, epididymis) are intravenously injected (16). This may explain why the rise of serum β -hexosaminidase in rats after zymosan injection is of short duration (14). In the present study the serum levels of β -hexosaminidase remained increased for at least 120 min over the basal levels. This could be explained by a secondary, Kupffer cell uptake of zymosan initially phagocytized by pulmonary macrophages (14).

Increased serum levels of the lysosomal enzyme lysozyme have been found in animals with an activated RES (17). The secretion of lysozyme is, however, not greatly changed by the presence of phagocytic material (9). Schrecker et al found that in mice, serum levels of lysosomal enzymes could serve as markers for macrophage activation (3). Tanner et al also found that both intracellular levels and

release of β -hexosaminidase were increased from liver macrophages isolated from *Corynebacterium Parvum* treated rats (18). In the present study, however, the plasma enzyme activity in rats pretreated for two days with zymosan did not differ from rats not pretreated.

Methyl palmitate is a well established suppressor of reticuloendothelial function in rats (8). We have shown suppression of RE function, measured as a reduction of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid liver uptake rate, in rats treated for two days with methyl palmitate (Rydén S, unpublished data 1983). A reduction in RE phagocytic capacity after trauma has been correlated with increased plasma levels of cathepsin and acid phosphatase in rats (19). One explanation for this observation has been a defective clearance from the blood due to a decreased Kupffer cell function (19). In the present study, the basal enzyme levels for methyl palmitate treated rats did not differ from those in control animals, so the hypothesis of a defective clearance seems unlikely.

Although resting macrophages secrete lysosomal enzymes (5) this secretion is increased during macrophage activation and phagocytosis (11, 12, 14). In trauma the RES is activated by numerous agents such as microthrombi, bacteria, endotoxin and damaged platelets (19), which may cause increased levels of lysosomal enzymes. In liver disease, RES may also be activated by enteric antigen by passing the filtering action of the liver (20).

The present study revealed significantly higher plasma levels of β -hexosaminidase after zymosan injection in rats with stimulated RES compared to rats with methyl palmitate suppressed RES. However, the enzyme response for the methyl palmitate pretreated animals did not differ significantly from that in control animals. This observation suggests that plasma levels of β -hexosaminidase after zymosan injection are more dependent on the degree of macrophage activation than on lysosomal enzyme clearance.

Alcohol administration did not influence the rise of β -hexosaminidase levels after zymosan injection in zymosan pretreated rats, nor in non-pretreated rats. Earlier studies in this laboratory have shown that alcohol alone given to rats does not influence plasma β -hexosaminidase activity (Rydén S, unpublished data 1983). These findings are not in accordance with earlier results concerning β -hexosaminidase activity in acute alcohol intoxication in man (2). Those patients had, however, ingested excessive amounts of alcohol for several weeks.

In conclusion, isolated measurements of plasma β -hexosaminidase activity in animals without concomitant macrophage challenge are of little value in predicting the status of the reticuloendothelial system. After a standardized zymosan injection, the level of plasma β -hexosaminidase activity may be correlated to the functional status of the RE system.

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INFLUENCE OF A RETICULOENDOTHELIAL-SUPPRESSING AGENT ON LIVER TUMOR GROWTH IN THE RAT

Running title: Reticuloendothelial suppression and experimental liver tumor growth.

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ABSTRACT

Reticuloendothelial function was evaluated by measuring the bio-kinetics of a standardized $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid. Methyl palmitate was administered intravenously on two consecutive days. A statistically significant reduction in the colloid uptake rate of the liver was registered after methyl palmitate administration. Histological examination revealed no signs of destruction of RE cells or micro-embolization.

Inoculation of an experimental nitrosoguanidine-induced, transplantable adenocarcinoma to the liver was performed in 16 rats one day after methyl palmitate administration and in 16 controls. Tumor size was significantly larger in methyl palmitate treated animals at 7 and 14 days after inoculation. Survival was significantly decreased in methyl palmitate treated rats. These rats showed signs of fatty vacuolization and necrosis of liver parenchyma earlier than controls.

Analyses of β -hexosaminidase and lactate dehydrogenase revealed no deviation of enzyme levels neither before nor after tumor inoculation.

The results indicate that a temporary suppression of RE function at the time of tumor inoculation may influence subsequent tumor growth.

KEY WORDS: Reticuloendothelial suppression; phagocytosis; Kupffer cells; liver tumor; tumor growth; methyl palmitate; colloids; scintillation camera and lysosomal enzymes.

INTRODUCTION

The macrophage represents one of the major effector mechanisms of the host in defense against tumor growth. Several experimental and clinical studies have shown macrophage activation in relation to tumor growth (1-5). The simultaneous administration of activated macrophages with tumor cells resulted in a significant inhibition of primary tumor growth as well as of metastases (6). Macrophage content of tumors in relation to metastatic spread and host immune reactions have also been studied (7, 8). Compared to extensive data regarding the in vitro cytotoxic effect of activated macrophages on tumor cells, little information is available regarding the role of fixed macrophages, i.e. the stationary part of the reticuloendothelial system (RES) (6). Several studies have been undertaken to establish the role of an activated RES in tumor growth. Glucan, a potent stimulant of RE function has been shown to inhibit experimental tumor growth (9). It has been established that surgery is followed by a short period of suppressed RE function (10). During RE suppression, a decreased resistance to tumor cell challenge has been noted (11). Methyl palmitate is a potent suppressor of the phagocytic activity of the RES (12). The phagocytic depression following methyl palmitate administration has been found to be primarily due to a selective macrophage impairment coupled with a slight reduction of opsonic activity (13). The administration of methyl palmitate has been shown to enhance the growth of a subcutaneous adenocarcinoma in mice (6).

A scintillation camera technique for measurement of the reticuloendothelial function has been demonstrated and shown to be reproducible (14). Applying this technique, alterations in RE function have been observed during different stages of tumor growth (15). Release of lysosomal enzymes has been correlated with the process of phagocytosis (16, 17) and intracellular levels of lysosomal enzymes have been shown to be increased in the liver of tumor-bearing mice (18).

The aim of the present study was to investigate the effect of methyl palmitate on RE function and on the growth of a transplantable adenocarcinoma in the rat liver. The suppressant effect of methyl palmitate on the RE function was measured with a $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid. Histological preparations of lung, liver and spleen were examined after methyl palmitate administration. Histological examinations of tumors and surrounding liver parenchyma were also performed during the course of the experiment.

A lysosomal enzyme, β -hexosaminidase (B-N-acetylglucosaminidase, EC 3.2.1.30), was evaluated to identify whether methyl palmitate or tumor growth either alone or in combination would affect the plasma enzyme levels. Lactate dehydrogenase (LD, EC 1.1.1.27) was assayed since LD release has been correlated with cell damage and cell death of elicited macrophages (17).

MATERIALS AND METHODS

Animals

In the whole experiment fifty-six male, inbred rats of Wistar strain maintained on standard food pellets and water ad libitum were used.

For studies of RE function, 20 rats (180-220 g) were divided into four groups (A-D). Five rats (A) were untreated controls and five rats (B) were injected intravenously with 1 ml 5 % dextrose with 0.1 % Tween 20 on two consecutive days (1 and 2). Ten rats (C, D) were injected with methyl palmitate 200 mg/100 g body weight, intravenously on days 1 and 2. All injections were made in the jugular vein with the animals under ether anesthesia. RE function was assessed on day 3 in groups A, B and C and on day 6 in group D.

Thirty-two rats, 310-400 g, were used for studies of tumor growth. Sixteen rats (controls) received an intravenous injection of 1 ml 5 % dextrose with 0.1 % Tween 20 on two consecutive days (1 and 2). Sixteen rats were given methyl palmitate, 200 mg/100 g body weight on days 1 and 2. All injections were made in the jugular vein during ether anesthesia. All animals were inoculated with a tumor cell suspension on day 3. Four rats were used for morphological studies after methyl palmitate administration.

Methyl palmitate

Methyl palmitate (Sigma Co., USA) was prepared as an emulsion by sonification for 10 min in 0.1 % Tween 20 in 5 % glucose solution (19). The concentration of methyl palmitate in the emulsion was 400 mg/ml.

Methods for determination of reticuloendothelial function

The test substance, $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid ($^{99}\text{Tc}^{\text{m}}_2\text{S}_7$) was prepared according to Persson and Naversten (20). The range of the particle diameter was 200 nm to 500 nm with a mean of 300 nm (21). The number of particles per ml solution was approximately 10^{10} . The labelling efficiency was tested in each experiment, with gel chromatography column scanning technique (22), and was found to be 97-100 %.

Under general anesthesia with Nembutal 6 mg/kg body weight, the jugular vein was cannulated and a catheter introduced to the superior vena cava. About 15 MBq in a volume of 0.5 ml test colloid containing approximately $0.5 \cdot 10^{10}$ particles was injected during 4-6 secs. Registration started immediately at the onset of injection.

For measurement of the kinetics of the activity distribution in the animals, a scintillation camera (Searle, LFOV, Searle Radiographic Inc.) was equipped with a parallel 39 500 hole collimator. From the

start of colloid injection sequential images were taken for 15 min. Three frame groups were stored with 16, 12 and 20 frames at intervals of 7.5, 15 and 30 secs, respectively. Regions of interest were selected in the image and time-activity curves were generated.

The time-activity curves were stored on a RK06 disc and transferred to a computer (Digital Equipment PDP 11/45) where the uptake curves were evaluated.

An open two-compartment model was used to estimate the flow rates of the radio-labeled colloid to the liver (k_1) and to other RE tissues (k_2) as shown in Figure 1. With the same symbols as in Fig 1 the formulas for the clearance of the colloid from the blood, B, and the rate of the uptake in the liver, L, are:

$$\frac{dB}{dt} = -B \cdot (k_1 + k_2) \quad (1)$$

$$\frac{dL}{dt} = B \cdot k_1 \quad (2)$$

The amount of colloid transported to the liver can be expressed by the equation:

$$L(t) = 100 \frac{k_1}{k_1 + k_2} \{1 - e^{-(k_1 + k_2)t}\} \quad (3)$$

The rate constants k_1 and k_2 were then obtained by fitting this equation to the experimental curves for each animal (14, 15).

Tumors

N-methyl-nitrosoguanidine-induced adenocarcinoma of the colon (NGW) was maintained on Wistar rats through serial transplantations in the kidney every 10 days (23). Under sterile conditions, a cell suspension was manufactured as described earlier (24). Vital cell counts were made in a hemocytometer after adding trypan blue. Within 1 hr after preparation, 0.1 ml (1.0×10^6 cells) of the suspension was inoculated into the periphery of the central lobe of the liver, just beneath the liver capsule. Access to the liver was gained through a midline abdominal incision, with the animal under light ether anesthesia. To avoid leakage of tumor cells, a spongostan sponge was pressed against the inoculation site until hemostasis was complete.

Measurements of tumor size were performed at 7, 14 and 18 days after tumor inoculation. With the animal under ether anesthesia, the

abdomen was reopened through the earlier incision. The tumors in the liver were measured with vernier calipers in the greatest (a) and the smallest (b) superficial diameters on the ventral side of the liver lobe. Tumor volume v (mm^3) was calculated using the approximation $v = a \cdot b^2/2$ (24). Extrahepatic tumor growth and ascites were identified.

Enzyme analyses

For enzyme analyses, 0.5 ml heparin-plasma samples were collected from the retroorbital plexus. Samples were taken before methyl palmitate/dextrose injection and before tumor inoculation. Samples were also taken at the time of tumor measurement from 8 animals of the control and 8 animals of the methyl palmitate treated groups. β -hexosaminidase and LD were analysed with methods described earlier (25, 26).

Morphological examination

Lung, liver and spleen samples were taken from animals on day 3 and day 6 after methyl palmitate injection. At each day of tumor measurements, one animal of each group was sacrificed, and samples of tumor and surrounding liver parenchyma were collected. All samples were fixed in 4 % formaldehyde, embedded in paraffin and stained with hematoxylin-erythrosine, the periodic acid-Schiff reaction and Turnbull blue-method for iron. Unembedded slices were stained with scarlet red for fat. The extent of necrosis, fat and iron pigment was evaluated from - to +++ (Table III).

Statistical analyses

Values of k_1 and k_2 for the compartment analyses were found by computing the single best-fit linear relationship as described earlier (14). When testing for differences in k_1 and k_2 and in tumor volume, Student's t-test was used. The Chi-2 test was used for comparing of survival between the two groups. When testing for differences in enzyme levels, Student's t-test and the non-parametric Wilcoxon rank sum test were used. A p value of less than 0.05 was considered significant.

RESULTS

Methyl palmitate-RE function

The results of the determination of RE function are depicted in Table I. The colloid uptake rate of the liver, k_1 , was statistically significantly reduced in rats receiving methyl palmitate and examined on day 3 (C). The uptake rate of the colloid into the extrahepatic RES, k_2 , was unchanged. The 5 methyl palmitate treated rats examined on day 6 (D) showed no significant differences in k_1 and k_2 compared to controls. No differences were noted between untreated animals (A) and animals treated with dextrose-Tween 20 (B). A scatter diagram of the rate constants is given in Figure 1.

Table 1. Uptake rate of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid in the liver (k_1) and the extrahepatic RES (k_2). p-values compared to control (A).

Groups	n	Colloid uptake rate (s^{-1})			
		Liver 10^{-2}	P	Extrahepatic RES $10^{-3} \cdot k_2$	P
A. Control, no treatment	5	1.8 ± 0.1		2.6 ± 0.2	
B. Tween 20 + dextrose	5	1.8 ± 0.1	n.s.	2.3 ± 0.5	n.s.
C. Methyl palmitate 3 d	5	0.62 ± 0.51	< 0.001	1.8 ± 0.6	n.s.
D. Methyl palmitate 6 d	5	2.0 ± 0.2	n.s.	2.3 ± 1.1	n.s.

n.s.: not significant at the 0.05 level

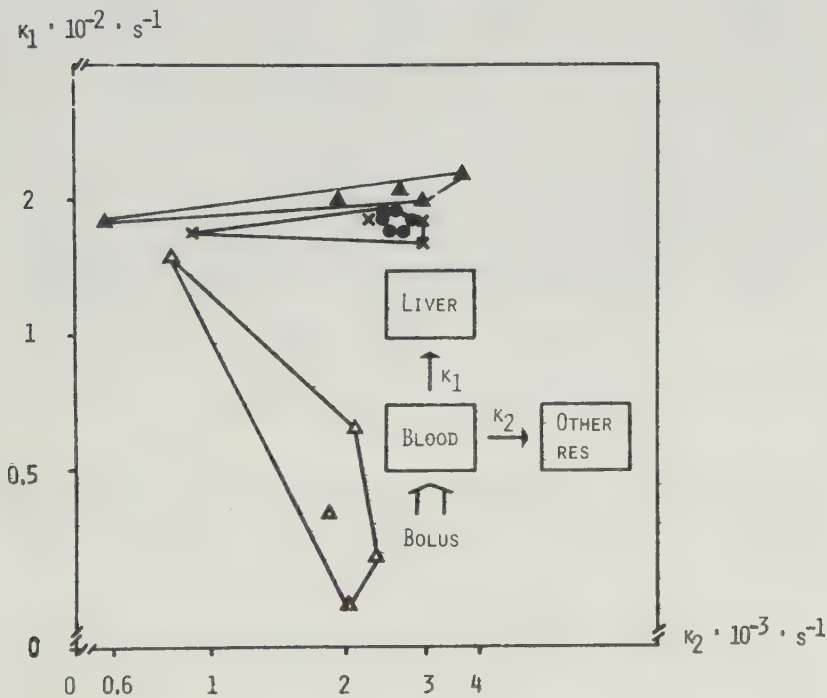


Fig 1. Scatter diagram of the rate constants k_1 and k_2 obtained from the two-compartment analysis. o = control, no treatment; x = control, Tween 20 + dextrose; Δ = methyl palmitate, 3 d prior to registration; $\blacktriangle$ = methyl palmitate 6 d prior to registration.

Tumor growth

All animals developed liver tumors. No spontaneous mortality was registered in the control group until day 18. In the methyl palmitate treated group only 4/14 survived until day 18. Survival in control animals was 19 ± 2 days (mean $\pm$ SD) and in methyl palmitate treated animals 15 ± 3 days. The difference was statistically significant with a p value of < 0.001 .

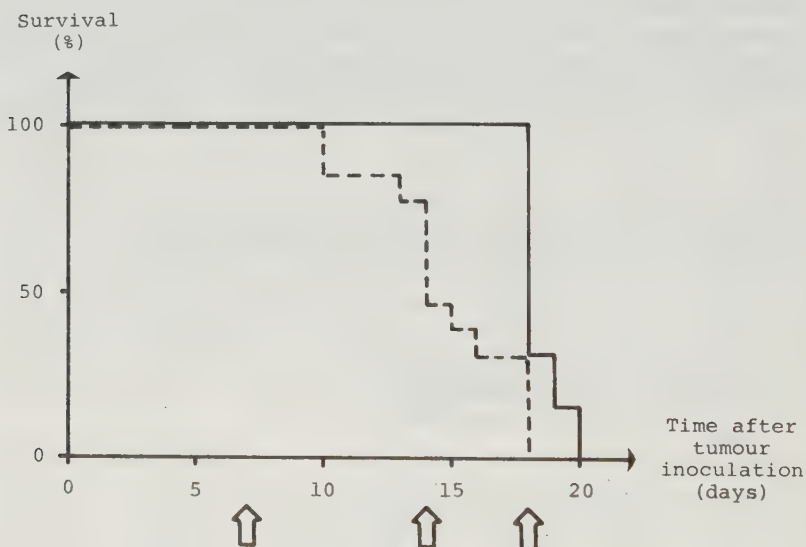


Fig 2. Survival for control rats (solid line) and for methyl palmitate pretreated rats (dotted line). Arrows indicate days of tumor examination.

A significant weight loss was observed in both groups during the first week after tumor inoculation, more pronounced in methyl palmitate treated animals (Fig 2). In the methyl palmitate treated group no further weight loss was noted. Control animals lost weight between day 14 and day 18.

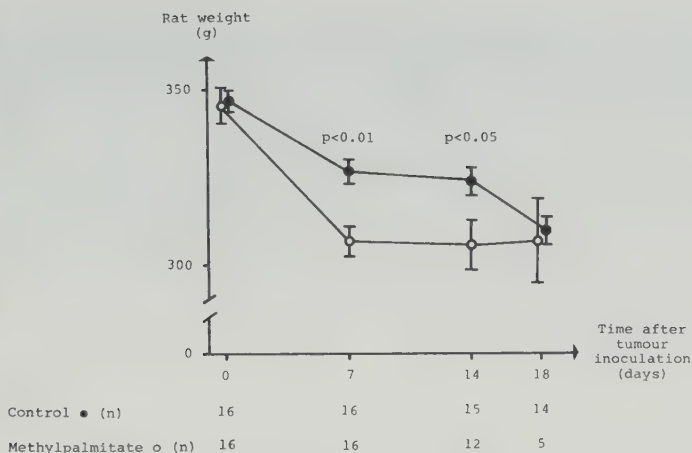


Fig 3. Weight of rats (mean $\pm$ SEM), during liver tumor growth. p values for comparison by Student's t-test.

Table II shows the logarithms of liver tumor volume for different times. A significant difference was noted in tumor size at 7 and 14 days, methyl palmitate treated rats having larger tumors. At day 18, no significant difference in liver tumor size between the two groups was noted.

Table II. Log tumor volume (mm^3) (mean $\pm$ SD) for measurements at 7, 14 and 18 days after tumor inoculation. p-values for difference according to Student's t-test.

	7 days		14 days		18 days	
	n	volume	n	volume	n	volume
Control	16	2.12 $\pm$ 0.20	15	2.97 $\pm$ 0.24	14	3.82 $\pm$ 0.20
Methyl palmitate	16	2.82 $\pm$ 0.27	12	3.54 $\pm$ 0.29	5	3.83 $\pm$ 0.05
		p < 0.001		p < 0.001		p n.s.

After 7 days 2/16 control animals and 6/16 methyl palmitate treated rats had signs of minor extrahepatic tumor growth confined to the abdominal wall. No animals showed signs of ascites or cachexia at this time.

After 14 days, all animals had signs of extrahepatic tumor growth. In the control group this was confined to the abdominal wall and none of these had ascites or expressed signs of malnourishment. All 12 surviving methyl palmitate treated rats appeared malnourished with a shaggy fur. At laparotomy all these revealed significant extrahepatic tumor growth with peritoneal carcinomatosis and ascites.

At day 18, all rats had developed these signs of extensive intra-abdominal tumor growth.

Blood samples for enzyme analyses were taken from every second animal in order to observe if the repeated blood sampling would influence tumor growth and the general condition of the animals. No such differences were, however, observed.

The enzyme levels of both β -hexosaminidase and LD revealed no changes after methylpalmitate or dextrose administration. Nor were any statistically significant changes in enzyme activity noted during the course of the experiment. It was not possible to detect any differences in the levels of β -hexosaminidase or LD between the two groups of animals during tumor growth.

Histological examinations after methyl palmitate administration revealed no signs of particle phagocytosis or microembolization in the lungs. A slight increase of fatty vacuolization in the liver parenchymal cells was detected at day 3 and one rat examined on day 6 (3 days after methyl palmitate administration) showed increased iron deposition in the phagocytes of the liver. No signs of liver necrosis were seen after methyl palmitate administration.

Table III. Histological evaluation of tumor and surrounding liver parenchyma 7, 14 and 18 days after liver tumor inoculation: Grading from - to +++, see text.

Days after tumor inoculation	LIVER Iron pigmentation in phagocytes		Lipid vacuoles in paren- chymal cells		Necrosis in paren- chymal cells		TUMOR Necrosis	
	Control	Methyl palm	Control	Methyl palm	Control	Methyl palm	Control	Methyl palm
7	(+)	(+)	-	-	-	-	+	+
14	+	+	-	+++	-	+++	++	+++
18	+	+	++	++	+	+	+++	+++

The results of the histological examinations of the tumor and surrounding liver parenchyma are depicted in Table III. Iron deposits in the phagocytes of the liver were present to the same extent in both groups. Lipid vacuolization and necrosis in the parenchymal cells of the liver were absent at day 7 in both groups but pronounced at day 14 in the methyl palmitate treated animals. At day 18 no differences were noted. Tumor necrosis was observed to a similar extent in both groups and increased with time.

DISCUSSION

The liver is the main stationary RE organ comprising about 80 % of the total fixed RE capacity (27). A depression of the RE function of the liver may therefore have a special bearing on the growth of tumors in the liver.

The method of assessing RE function has shown to be reproducible in earlier studies (14⁹⁹, 15). It has the advantage of non-invasive measurements. The $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid particles used in the experiments have been characterized in respect of size, shape, number and labelling efficiency (20-22).

The RE suppressant effect of methyl palmitate has been established by other investigators (12, 13) and is confirmed in the present study. This effect seems to be due to a depression of hepatic RE function. The pronounced decrease of the hepatic RE function does not coincide with a change in phagocytic capacity of the extrahepatic RES. In previous studies of the RE function after methyl palmitate administration (6, 12, 13), no distinction between the hepatic and the extrahepatic RE functions was made. The effect of methyl palmitate on the hepatic RES seems to be of short duration since no changes in hepatic RE function could be observed on the sixth day. This is in agreement with DiLuzio's earlier findings about the duration of the RE blocking effect of methyl palmitate (6).

The histological examinations revealed no signs of destruction of RE cellular elements after methyl palmitate infusion. These results are in accordance with similar observations on mice (12). Administration of methyl palmitate did not influence the levels of β -hexosaminidase and LD as measured at the day of tumor inoculation. This may indicate that the clearance of β -hexosaminidase by Kupffer cells was not influenced by methyl palmitate. Methyl palmitate did not cause any release of LD, signifying no excess cell damage and cell death. These findings were in accordance with the morphological examination which did not show any necrosis in the liver or lung after methyl palmitate treatment.

The effects on the growth of an experimental liver tumor by hepatic artery ligation and hyperthermia have been reported from this laboratory (24, 28). By injecting a tumor cell suspension into the periphery of the central liver lobe just under the capsule, it was possible to produce a single ellipsoid liver tumor. In the experimental situation, hematogenous tumor cell injection would give rise to multiple tumors making tumor size calculations more hazardous. The simplified formula for estimating tumor volume has been shown to be reliable and well correlated with the actual ellipsoid tumor volume (24). Earlier experience with the same liver tumor showed that more than 3 laparotomies in the 3 week post-inoculation period were associated with a high mortality of rats (24). Inoculation of liver tumors was performed at a time of significant hepatic RE depression as revealed by the reduced hepatic uptake rate of $^{99}\text{Tc}^{\text{m}}$ -sulphur colloid. Other factors may influence RE phagocytic activity, such as numbers and duration of anesthetic procedures, laparotomies and blood sampling (10, 27). These factors were carefully held equal in both methyl palmitate treated and control animals. Because of a small number of surviving rats in the methyl palmitate treated group at day 18, no conclusion regarding statistically significant differences could be made at that time.

Striking differences concerning tumor growth, the general condition of the animals and survival were noted between the two groups. It thus seems that a hepatic RE depression of even short duration can have profound effects on the development of liver tumors. The liver is the most common site of secondary tumor localization originating from gastrointestinal primary tumors. This occurs in spite of the fact that the liver contains the majority of the stationary RE cells (27). The function of these cells as host defense may be abrogated by several physiologic and pathophysiologic factors (6, 10, 11, 14, 27, 31) and the present data underlines that a hepatic RE depression even of short duration can have profound effects on the initial course of liver tumors.

The observed enhancement of tumor growth was in accordance with observations of accelerated growth of a subcutaneous adenocarcinoma in mice after methyl palmitate administration (6). This early suppression of macrophage function may be of importance for subsequent tumor development (29). In a study of the development of experimental lung metastases in Wistar rats, trauma enhanced the development of lung metastases. The combination of trauma and dextran 40 were even more effective in this respect (30). These factors are known to depress RE function alone or in combination (31). Clinical experience indicates that patients with non-resectable carcinoma often demonstrate rapid deterioration and accelerated tumor growth after surgery. The initial RE suppression, seen after surgical procedures (10, 27) may be a factor of some importance in this context.

Increased serum levels of lysosomal enzymes have been observed in animals with an activated RES (32). It has been proposed that serum

levels of lysosomal enzymes could serve as markers for macrophage activation (33). Biochemical studies have revealed significant increases in acid phosphatase, cathepsin-D and β -galactosidase in hepatocellular lysosomes of mice bearing the solid or ascites form of sarcoma 180 (18). β -hexosaminidase secretion from activated peritoneal macrophages were increased during phagocytosis (17). Elevations of serum levels of β -hexosaminidase have been observed in patients with liver disease (25). It was, however, not possible in the present study to show any difference at the 0.05 level of significance in plasma levels of β -hexosaminidase in response to tumor growth, nor was any difference apparent after methyl palmitate RE-suppression. A difference may, however, exist between different lysosomal enzymes since glucan activated hepatic macrophages showed increased levels of especially lysozyme while β -hexosaminidase was unaffected (34). Whether other lysosomal enzymes may be affected has to be tested in further studies.

LD release is usually considered to be an indication of cell damage and death. The release of β -hexosaminidase of elicited macrophages were not combined with LD release (17). In this study, LD release was not apparent in spite of histological evidence of necrosis of tumor and liver parenchyma.

CONCLUSION

With the applied technique of RE measurements it has been possible to demonstrate alteration of colloid uptake rate after methyl palmitate treatment. The striking effects on tumor growth of a short period of RE depression manifested by the decreased hepatic colloid uptake rate induced by methyl palmitate indicates the importance of the macrophage system in host defense against tumors.

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